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UNIVERSITY OF ALBERTA

DEVELOPMENT OF A SYNTHETIC PEPTIDE ANTI-*PSEUDOMONAS AERUGINOSA* ADHESION VACCINE

KOK KHEONG LEE

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

IN

DEPARTMENT OF BIOCHEMISTRY

EDMONTON, ALBERTA

SPRING, 1991

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FACULTY OF GRADUATE STUDIES AND RESEARCH

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ABSTRACT

The occurrence of opportunistic *Pseudomonas aeruginosa* infections in hospitals represents a major problem. Antibiotic treatment is inadequate in the complete eradication of these organisms. The adherence of *P. aeruginosa* to host cells which is mediated by pili represents the initial stage in bacterial infections. This thesis describes the development of an anti-adhesion *Pseudomonas* vaccine. The pilus is a filamentous structure consisting of a polymer of a single protein subunit called pilin (Mr ~ 15kDa). Synthetic peptides corresponding to predicted surface regions of *P. aeruginosa* PAK and PAO pilins (144 residues in length) were used to raise antipeptide antibodies. Eight potential surface regions were predicted and most of the antipeptide antibodies to these regions bound to native PAK and PAO pilins. Four of the anti-PAK peptide antisera crossreacted with strain PAO pili, while five anti-PAO peptide antisera crossreacted with strain PAK pili. Only one region of the two pilin proteins (region of residues 88-97) provided strain-specific antipeptide antibodies. The results emphasized the complexity of the immune system and the difficulty of predicting regions of a protein molecule which could give rise to strain-specific and crossreactive antibodies.

Unlike the gonococcal pilin, the disulfide bridge region of PAK pilin (residues 128-144) does not contain the immunodominant strain-specific epitopes. Of all the antipeptide antibodies prepared, the region of residues 128-144 of both PAK and PAO pilins provided the best binding antibodies. Polyclonal antipeptide antibodies and monoclonal antibodies directed against this region blocked the adherence of PAK pili to human buccal epithelial cells (BECs). Although the integrity of the disulfide bridge was not critical in raising antibodies, it was important for raising crossreactive antibodies. This region of pilin contains the receptor-binding domain. Interestingly, under the appropriate conditions, it was found that a longer C-terminal peptide (residues 116-144) could enhance the binding of PAK to BECs if the disulfide-bridge was intact, showing the importance of the bridge

in maintaining the function of this binding domain. The shorter AcPAK 128-144 peptide bound to the cell-surface receptors in a concentration-dependent, saturable manner and also competitively inhibited PAK pili binding, suggesting the occupation of the same receptor site. This region represents a good candidate for an anti-adhesive *Pseudomonas* vaccine.

In the course of these studies, two promising applications were developed. It was shown that PAK 128-144 peptide could be conjugated to a reporter moiety (alkaline phosphatase) using the photoactivated benzoylbenzoyl group, and this conjugate could be used to visualize binding of the peptide to BEC receptors. This procedure could be used to study peptide-protein or peptide-receptor interactions. One problem encountered in the formation of an intrachain disulfide bridge is the availability of a rapid and simple monitoring method to follow the extent, and quantitation, of the oxidation process. Thus, a second application that was developed involved the use of reversed-phase chromatography to obtain baseline resolution of reduced and oxidized peptides and quantitate the conformers after modification of the reduced peptides with a thiol-specific reagent, N-ethylmaleimide.

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List of Abbreviations

ABTS	2,2'-azido-di(3-ethylbenzthiazoline sulfonic acid)
A_{405}	absorbance reading taken at 405nm
Ac	acetyl
AP	alkaline phosphatase
aq	aqueous
BCIP	5-bromo-4-chloro-3-indolylphosphate-toulidine
BECs	human buccal epithelial cells
BSA	bovine serum albumin
Boc	*tert*-butyloxycarbonyl
Bzl	benzyl
C-8	octyl ligands on RPC column
C-18	octadecyl ligands on RPC column
CD	circular dichroism
DCM	dichloromethane
DCC	dicyclohexylcarbodiimide
DCU	N,N'-dicyclohexylurea
DIEA	diisopropylethylamine
DMF	dimethylformamide
g	gram
HF	hydrogen flouride
h	hour
HFBA	heptafluorobutyric acid
HOBt	1-hydrobenzotriazole
HPLC	high-performance liquid chromatography
I.D.	internal diameter
IEC	ion-exchange chromatography
IgG	immunoglobulin of the G class
KLH	keyhole limpet hemocyanin
MAb	monoclonal antibody
MEP	mucoid exopolysaccharide
mg	milligram
min	minute
Mol. Wt.	molecular weight
NBT	*p*-nitro blue tetrazolium chloride

NEM	N-ethylmaleimide
NH_2-	amino group
OBzl	benzyl ester
PAK	*Pseudomonas aeruginosa* strain K
PAO	*Pseudomonas aeruginosa* strain O
PBS	sodium phosphate buffered saline
PBST	sodium phosphate buffered saline containing 0.05% Tween 20
RPC	reversed-phase chromatography
SDS-PAGE	sodium dodecylsulfate polyacrylamide gel electrophoresis
SEC	size-exclusion chromatography
SPPS	solid-phase peptide synthesis
TEC	tracheal epithelial cell
TFA	trifluoroacetic acid
TBS	tris buffered saline
TTBS	tris buffered saline containing 0.05% Tween-20
UV	ultraviolet
ΔRt	difference in retention times
µm	micron
2Cl-Z	2-chlorobenzoxycarbonyl

Glossary

Adhesin	microbial surface antigens or molecule that bind to specific receptors on epithelial cell membranes
Alginate	a colloid acid polysaccharide (mucoid exopolysaccharide) consisting of a polymer of D-mannuronate and L-guluronate
Antibody	immunoglobulin, immune or protective proteins
Antigen	immunogen, any substance that induces an immunogenic response
Antigenicity	the ability of a particular molecular structure to be recognized by B-cell (antibody) or T-cell receptor molecules
Antigenic determinants	particular chemical group of a molecule that confers antigenic specificity (synonymous with epitope)
Antibody titer	the lowest dilution of the serum that can still detect the presence of antibodies raised against an antigen
Arthritis	inflammation of a joint
Bacteremia	the presence of viable bacteria in the circulating blood
Bronchiectasis	chronic dilation of bronchi or bronchioles as a sequel of inflammatory disease or obstruction
Crossreacting antibodies	antibodies that have functional groups of closely similar, but not, identical chemical structure
Fimbria	hair-like structure protruding on the surface of gram-negative and gram-positive bacteria, made up of protein subunits arrayed as a filamentous structure; pilus (pl. fimbriae)
Hapten	a molecule (partial antigen) that is incapable, alone, of causing the production of antibodies but is capable of combining with specific antibodies; can generate anti-hapten antibody production when conjugated to a protein carrier molecule
Immunocompromised	denoting an individual whose immunological mechanism is deficient either because of an immunodeficiency disorder or because it has been rendered so by immunosuppressive agents
Immunogenicity	the ability of a molecule to induce an immune response
Immunoglobulin	one of a class of structurally related proteins consisting of two pairs of polypeptide chains, one pair of light and one pair of heavy chains; in the case of immunoglobulin G, all four chains are linked together by disulfide bonds
Immunoprophylactic	an antigenic (immunogenic) agent which actively stimulates the immunological system to prevent a disease

Immunosuppressed	prevention or interference with the development of immunologic responses, may be artificially induced or caused by disease
Infect	to enter, invade another organism causing infection
Leucocytosis	an abnormally large number of leukocytes, as observed in an acute infection
Metastasis	spread of a disease process from one part of the body to another
Morbidity	a diseased state
Mortality	pertaining to death, a fatal outcome
Mucoid	mucous secretion
Necrosis	pathologic death of a cell, part of tissue or organ resulting from irreversible damage
Neutropenia	abnormally low number of neutrophils (mature white blood cell, granulocyte) in the circulating blood
Nosocomial	hospital-acquired infection
Occlusion	state of being closed
Pathogenicity	ability to cause disease
Pilin	protein subunit which assemble by subunit-subunit interactions to form a filamentous structure (pilus)
Pilus	a fine filamentous appendage similar to fimbria(pl. pili)
Peritrichous	cilia or appendages protruding from the periphery of a cell
Pyrexia	fever
Pyocyanin	blue pigment secreted by *Pseudomonas aeruginosa*
Septicemia	systemic disease caused by the spread of microorganisms and their toxins via the circulating blood
Thrombosis	clotting within a blood vessel
Vaccine	any preparation (e.g., killed viruses;microbial products) intended for active immunological prophylaxis
Virulent	extremely toxic, denoting a markedly pathogenic microorganism

Chapter I. Introduction

A. Introduction

Pseudomonas aeruginosa, a species of the genus *Pseudomonas*, is characterized by the production of a blue green pigment, pyocyanin, which is a phenazine. The organism is a gram-negative, unicellular rod of dimensions of 0.5 to 1.0 μm by 1.5 to 4.0 μm. The physiological characteristics of *P. aeruginosa* include simple growth requirements, utilizing a large number of organic compounds for growth. It can also thrive on mineral water with only ammonium salts as a nitrogen source. The organism thrives in a moist environment. The bacterium exhibits an active swimming motility which is mediated by a polar flagellum. A twitching movement produced by these organisms has been observed on the surface of solid media. This twitching motility was observed under light microscopy in non-flagellated strains of *P. aeruginosa* (Feurst and Hayward, 1969) and polarly inserted pili were responsible for this motion. While the *P. aeruginosa* pili are polarly located, there are also peritrichous (protruding from the side) pili found in species of *P. cepacia* and *P. fragi* (Palleroni, 1975).

B. Pseudomonas infection

P. aeruginosa is classed as an opportunistic pathogen. It does not cause disease in healthy individuals but poses a serious threat to immunocompromised or immunosuppressed patients. *P. aeruginosa* is sometimes present as part of the normal microbial flora of humans. However, the prevalence in healthy individuals is very low, being in the range of a few percent only (Pollack, 1990). Opportunistic organisms have characteristically low pathogenicity and usually cause serious infections only when the host defense mechanisms are impaired. Factors which predispose patients to gram-negative bacilli infections include administration of broad spectrum antibiotics such as ampicillin. The carrier rate of *P. aeruginosa* among patients who received antibiotics was found to be

31%. *P. aeruginosa* was only isolated from 11% of the people who had not received antibiotics (Bodey *et al.*, 1983). Such treatments eliminate the normal bacterial flora, enabling incoming opportunistic organisms to colonize and multiply. In addition, instrumentation and surgical procedures also create opportunistic conditions for such infections. Thus, the intensive care units, burn units and oncology units report a high incidence of these infections. Impaired host defense mechanisms can be exhibited by patients with acute leukemia or cancer. These patients are frequently neutropenic as a result of their disease or related therapies. The neutropenic condition makes the patients very susceptible to *Pseudomonas* infection. The neutrophils, monocytes and macrophages play an important role in host defense cell-mediated immunity against *Pseudomonas* infections. Thus, one can see that *P. aeruginosa* is a major problem in nosocomial infections. The longer a patient remains in the hospital, the greater the likelihood of colonization by *P. aeruginosa*. The risk of infection is substantially greater among patients if they have some defect in their defense mechanism. In a survey conducted in 1983 by the Center for Disease Control in Atlanta, *P. aeruginosa* was one of the four most common nosocomial pathogens in hospitals in the United States (Jarvis *et al.*, 1984). This accounted for 9.9% of all nosocomial infections. It is the second leading cause of nosocomial pneumonia (13.1% of all pneumonia), the third most common cause of urinary tract infection (11.7%), the fourth leading cause of primary gram-negative bacteremia and the highest mortality rate associated with gram-negative bacteremia. The frequency of hospital incidence is on the rise; from 6.6% in a 1976 survey to 9.9% in the 1980-1982 survey (Pollack, 1990). *Pseudomonas* infection in cystic fibrosis (CF) patients also represents a major threat. The reported rates of colonization have varied from 18 - 80% (Bodey *et al.*, 1983). *P. aeruginosa* has been the most common isolated *Pseudomonas* species isolated from CF patients (about 80 %), but lately there have been reports of an increasing number of *P. cepacia* detected in the sputa from these patients (Prince, 1986). Ninety percent of the mortality and most of the morbidity in CF patients is due to pulmonary dysfunction. While

pulmonary infection is secondary, it is the principal cause of irreversible damage to the lungs of CF patients. The colonization of the lungs of CF patients by these organisms is seldom, if ever, eradicated by intensive antibiotic therapy. However, the lifespan of CF patients is increased by such treatment.

There are two classes of *P. aeruginosa* clinical infections. Firstly, there are the localized infections where the organisms colonize and infect wounds or burns, urinary tract, respiratory tract, intestine, eye and ear; secondly, there are the generalized infections which arise from a primary local infection which then leads to the establishment of metastatic foci in various organs as the blood-borne organisms invade walls of small vessels producing inflammatory arthritis and thrombosis, arterial occlusions and development of small areas of necrosis. The septicemic spread results in neutrophil leucocytoses, pyrexia or hypothermia. The prognosis is poor and the mortality rate is high.

C. *Pseudomonas* virulence

The pathogenicity or virulence of an organism describes its capacity to cause disease. To be pathogenic, the organism must be able to (i) adhere and colonize the mucosal surfaces of the host, (ii) penetrate the mucous surfaces, (iii) establish and multiply in the environment of the host's tissues, (iv) resist or interfere with host defense mechanisms that try to remove or destroy them, and (v) cause damage to tissues of the host (Smith, 1984). Hence, microbial pathogenesis is complex and multifactorial as the organism goes through many phases before the production of a diseased state. Removal of one of the factors involved may render the organism less virulent or avirulent (Finlay and Falkow, 1989). Not unlike other bacteria, the pathogenicity of *P. aeruginosa* arises from the various virulence factors the organism produces. A number of criteria which are useful in the consideration of virulence factor expressed by *P. aeruginosa* have been discussed by Lory and Tai (1985). These criteria include:

i) the ability of the components to mimic one of the pathological effects of *Pseudomonas* infection when administered to animals in purified or partially purified form

ii) the injection into an animal eliciting at least a partial protective immune response against subsequent challenge with virulent strains of *P. aeruginosa*

iii) mutations that abolish the synthesis of a particular protein markedly decreasing the virulence of the organism.

The virulence factors, consisting of secreted exoproducts and components associated with the cell surface are tabulated in Table I.1. Exotoxin A (ETA) and exoenzyme S (ETS) are both ADP-ribosylating enzymes with different substrate specificity. ETA has elongation factor 2 as its substrate (Iglewski *et al.*, 1975), while ETS seems to ADP-ribosylate a number of cytoplasmic proteins, including vimentin (Iglewski *et al.*, 1975; Coburn *et al.*, 1989). *P. aeruginosa* secretes a number of proteolytic enzymes. Morihara and coworkers (Morihara, 1964; Morihara *et al.*, 1965) discovered an elastase which showed broad proteolytic activities against various proteins. The *Pseudomonas* elastase has been shown to degrade IgA and IgG, inactivate proteinase inhibitors, degrade basement membranes (Bejarano *et al.*, 1989) and inhibit human natural killer cell activity (Pedersen and Kharazmi, 1987). An alkaline protease was also found in *P. aeruginosa* (Morihara, 1963). This protease can also inhibit natural killer cell activity and degrade human gamma interferon (IFN-γ), a reaction which is augmented by elastase (Horvat *et al.*, 1989). Leukocidin (cytotoxin) destroys polymorphonuclear leukocytes (PMN) which represent the primary host defense mechanism against *P. aeruginosa* infections. Two hemolysins are produced by *P. aeruginosa*. Phospholipase C (PLC) hydrolyses phosphatidylcholine, a major phospholipid component of the lung surfactant, into diacylglycerol and phosphocholine . PLC can cause paralysis, death, dermonecrosis, footpad swelling and vascular permeability in mice (Berk *et al.*, 1987). A heat-stable glycolipid also has

TABLE I.1 Virulence Factors of *P. aeruginosa*

Virulence Factor	Effects	References
Exotoxin A	ADP-ribosylation of elongation factor 2 (EF-2)	Iglewski & Kabat, 1975. Van Ness *et al.*, 1980
Exoenzyme S	ADP-ribosylation of cytoplasmic proteins (other than EF-2) including vimentin	Iglewski *et al.*, 1978
Elastase	Broad specificity protease, degrades immunoglobulins A and G, inactivates proteinase inhibitors, degrades basement membranes	Morihara, 1965 Bejanaro *et al.*, 1989
Alkaline Protease	Degrades human gamma interferon (IFN-γ), inhibits human natural killer cell activity	Morihara, 1963 Pedersen & Kharazim, 1987
Leukocidin	Destroys polymorphonucleocytes, inhibits macrophage-mediated phagocytosis	Hirayama and Kato, 1984; Kluftinger *et al.*, 1989
Hemolysin (Phospholipase C)	Causes paralysis, death, dermo-neucrosis footpad swelling and vascular permeability in mice	Berk *et al.*, 1987
Hemolysin (Glycolipid)	Lysis of erythrocyte membrane	Kurioko & Liu, 1967
Pyocyanin	Inhibition of T-lymphocyte proliferation, interference of oxidative burst of phagocytes	Muller *et al.*, 1989 Ulmer *et al.*, 1990
Ferripyochelin binding protein (FBP)	Aids uptake of iron by taking up Ferripyochelin	Sokol, 1987
Pilus	Adherence of bacterium to epithelial cell surface	Woods *et al.*, 1989; Doig *et al.*, 1988
Lipopolysaccharide (LPS)	Endotoxin	Cryz *et al.*, 1983a; Cryz *et al.*, 1983b
Exopolysaccharide	Protects against phagocytes, adherence of bacterium to epithelial cell surfaces	McEachran & Irvin, 1985; Ramphal *et al.*, 1984

hemolytic ability and it is thought that its mechanism of action involves intercalation in erythrocyte membranes (Kurioko and Liu, 1967). *P. aeruginosa* also secretes iron scavengers such as, siderophores, pyochelin and pyoverdin, which can bind to siderophore-binding proteins found on the outer membrane. These binding proteins may be considered virulence factors, since mutants lacking these proteins are less pathogenic and are cleared more rapidly in the mouse corneal infection model (Sokol, 1987).

Some of the virulence factors associated with the cell surface include lipopolysaccharide (LPS), alginate (or exopolysaccharide) and pili. The LPS is an endotoxin and causes tissue damage. The alginate is a polymer of 1-4 linked D-mannuronic and L-guluronic acid (Linker and Jones, 1961) and gives rise to capsule formation which protects against phagocytes and act as an adhesin. The pili, which protrude from the ends of the rod-shaped bacterium, are filamentous structures which act as adhesins, anchoring the bacterium to cell surfaces.

D. Treatment for Pseudomonas infection

Antibiotic treatment is available for *P. aeruginosa* infections. *P. aeruginosa* has low susceptibility to antibiotics and usually a combined chemotherapy treatment is used, consisting of aminoglycosides and an extended-spectrum penicillin. Aminoglycosides are highly charged molecules and examples include gentamicin and streptomycin. The β-lactam drugs have been the most commonly used anti-*Pseudomonas* reagents because of their low toxicity and high activity. However, *P. aeruginosa* affects patients who are already in a debilitated state and the high resistance of this organism towards chemotherapy still results in high patient fatality rates. It is believed that there are three pathways through which antibiotics are taken up across the outer membrane of *P. aeruginosa*. Firstly, the hydrophilic antibiotics are believed to pass through water-filled pores of the outer membrane protein (OMP-F). A second pathway involves the partitioning of hydrophobic antibiotics across the membrane. This pathway is not expected to be of major importance

(Moore *et al.*, 1986). The third pathway is called the self-promoted uptake pathway (Moore *et al.*, 1986). Aminoglycoside, polymyxin B and other polycations are thought to interact with a divalent cation site on LPS which distrupts the membrane, enhancing the permeability of the membrane to these compounds. As mentioned earlier, *P. aeruginosa* has high resistance to antibiotics. One mechanism of resistance is the occurrence of plasmid-associated drug-modifying enzymes such as β-lactamase or aminoglycoside-modifying enzymes (acetyltransferase, phosphoryltransferases). A second form of resistance involves altered permeability to β-lactam drugs. The porin protein that is absent or mutated in *P. aeruginosa* fails to form functional channels for the transport of these drugs across the outer membrane of *P. aeruginosa*. There are a number of ways to enhance the efficacies of antibiotic treatment. One of these is to increase the outer membrane permeability. The combined aminoglycoside and penicillin treatment is effective because it is believed that the aminoglycoside enhances the uptake of penicillin by permeabilizing the membrane. The concept of using permeabilizers has been elaborated by Hancock (1986). Other possible methods of enhancing the efficacies of antibiotics include lowering the expression of drug-modifying enzymes and making the drugs more lipophilic to bypass the specific transporters and enter via the lipid bilayer. A new class of antibiotic, the quinolones, is proving to be effective against *P. aeruginosa*, although the exact mechanism of action of these drugs is not yet known.

E. Immunoprophylactic approach to *P. aeruginosa* treatment

The increase in the number of nosocomial cases in hospitals due to gram-negative bacteria (*E. coli*, *Klebsiella* spp and *P. aeruginosa*), the inadequacies of chemotherapy and the increasing incidence of antibiotic resistant strains highlight the need for an immunoprophylactic approach to the control of *P. aeruginosa* infections. This prophylactic treatment would provide a primed host defence mechanism against the infection. However, in situations where the patients have not been previously vaccinated, such an approach

requires too much time to mount a defense against *P. aeruginosa* and active immunization may be of limited value in these cases. Thus, passive immunization should be considered, whereby hyperimmune immunoglobulins isolated and purified from plasma of immunized volunteers could be used to treat *P. aeruginosa* infections. As with any effective vaccines, vaccines designed to treat *P. aeruginosa* infections need to elicit neutralizing antibodies which will assist in the eradication of *P. aeruginosa*. Humoral immunity and phagocytes represent the main components of protection against *P. aeruginosa*.

Surveys of a number of experimental vaccines against *P. aeruginosa* were carried out by Lieberman (1985) and Holder (1988) and some of these vaccines are shown in Table I.2. We have seen from Table I.1 that *P. aeruginosa* produces quite a number of somatic (cell-associated) or extracellular virulence factors. Antibodies directed against these virulence factors have been found to provide varying degrees of protection against experimental *P. aeruginosa* infections. The correlation of survival from an episode of *P. aeruginosa* septicemia with antibody levels to the various antigens have led some researchers to develop anti-toxin A and anti-LPS vaccines, since the levels of titers of antibody to the LPS and extoxin A were highest in the patients with the highest survival rates (Pollack and Young, 1979). The heptavalent lipopolysaccharide (LPS) (Pseudogen, Parke-Davis, Detroit, Michigan) and the 16-valent LPS (PEV-01, Wellcome Biotechnology Ltd, Beckenham, England) both provided varying degrees of protection in patients. The Pseudogen and PEV-01 vaccines are pooled surface antigen extracts from the different serotypes of *P. aeruginosa*. These vaccines have endotoxic side-effects, due to possible contamination of virulence products. The exact nature of protective antigen in the surface extract of PEV-01 is still unclear, although the LPS seems to be the dominant antigen present (MacIntyre *et al.*, 1986). Because of the endotoxic nature of the LPS-based vaccines, Pier *et al.* (1978) have tried to overcome this problem with the use of high molecular weight polysaccharide (PS), which is immunogenic but lacks the toxicity portion of LPS. This 'O' specific-like PS can be made more immunogenic by conjugation to a

TABLE I.2 *Pseudomonas aeruginosa* vaccines

Vaccine	Effectiveness	Problems	Reference
Hepta valent lipopolysaccharide (Pseudogen, Parke-Davis Co.)	Active and passive immunity in burn patients but moderate to no success in CF, cancer, and patients with respiratory failure	Endotoxic complications	Alexander *et al.* 1969
16-valent lipopolysaccharide (PEV-01, Wellcome Biotechnology Inc.)	Similar to Pseudogen	Endotoxic complications, nature of protective antigen unknown	Miler *et al.*, 1977
High and low molecular weight polysaccharides	Provided active and passive immunity in normal and burn mice models	Low immunogenicity, less protective than LPS vaccine, variation in mouse strain response to vaccine	Pier, 1982; Tsay and Collins, 1984
Low molecular weight polysaccharide - toxin A conjugate	Limited active and passive immunity in mice with serotype-specific and anti-exotoxin A antibodies		Cryz *et al.*, 1986
Multi-component vaccine (Toxoid alkaline protease, elastase and "original endotoxin protein")	More effective than single component in corneal infection in mice, mixed results in immunization in mink	Effectiveness of multi-component *vs* single component in animal models has mixed results in hemorrhagic pneumonia and burn wounds	Hirao and Homma, 1978; Homma *et al.*, 1978
Exotoxin A	Active and passive immunity in burn mice produced mixed results ranging from extension of survival time to no effect with passive therapy	Augmentation with antibiotic to produce good neutralization	Pavloskis *et al.*, 1977; Pavloskis *et al.*, 1981

Flagella	Protection in burned mice with flagella antigen-specific and non-specific O-serotype antibodies	Possible contamination with LPS	Holder *et al.*, 1982; Holder *et al.*, 1986
Elastase	Good protection in corneal challenge in rabbit but not in mice	Mixed results in different animals	Kreger *et al.*, 1986
Outer membrane protein (Porin F)	Active immunity even with heterologous challenge in normal and burn mice	Problem of LPS contamination	Greer-Matthews *et al.*, 1987; Gilleland *et al.*, 1984
Temperature-sensitive *P. aeruginosa* mutants	Active immunization in mice, enhanced lung clearance of homologous strains	Possible reversion of mutants to normal strains	Hooke *et al.*, 1987
Ribosomal vaccine	Active and passive immunity in normal and burn animal models	LPS contamination; true nature of protective antigen unclear	Lieberman *et al.*, 1986

The data in this table were derived from Lieberman (1985) and Holder (1988).

carrier protein. In fact, Cryz and coworkers (Cryz, 1987; Cryz *et al.*, 1987) have conjugated the PS moiety to exotoxin A. This detoxified the ETA and immunization with this O-polysaccharide-toxin A conjugate invoked an immune response to both LPS and ETA.

A number of protein-based vaccines that have been studied include the outer membrane protein (OMP-F), flagella, ETA, elastase and alkaline proteases. Omp-F is a protein which is antigenically related in all O-serotype strains of *P. aeruginosa*. Active immunization with OMP-F and passive immunization with polyclonal serum have provided protection in normal and burned mice (Greer-Matthews and Gilleland, 1987; Gilleland *et al.*, 1984). However, these proteins are difficult to obtain in pure form because of LPS contamination. A multicomponent vaccine comprised of toxoided alkaline protease, elastase and "original endotoxin protein" (OEP - a common protective antigen that could be used to prevent infections due to *P. aeruginosa*, regardless of the O-serotype of the infecting strain) has been reported by Hirao and Homma (1978) to provide active and passive immunity in experimental corneal infection in mice. A *P. aeruginosa* ribosomal vaccine has been examined by Lieberman *et al.* (1986) and found to be protective in normal mice and burned rats. However, LPS contamination in the ribosomal preparation and the exact nature of the protective antigen is unclear as trypsin- and ribonuclease- treated ribosomal vaccine also afforded protection.

As pointed out by Baltimore (1985), a successful vaccine would be one that would prevent the initial colonization. To this end, some researchers have been looking at alginate or mucoid exopolysaccharide (MEP) vaccines. This exoproduct, secreted by *P. aeruginosa*, consists of a linear heteropolymer of D-mannuronic and L-guluronic acids. Some of the D-mannuronate residues are mono- or di- acetylated. In the presence of divalent cations, the acetylated mannuronate residues form viscous large volume matrices around the bacterial cell wall in which the organisms can grow as microcolonies has been shown in the lung of CF patients (Lam *et al.*, 1980). Alginate acts as one of the two

known adhesins (the second is pili) of *P. aeruginosa* which mediates the adherence of mucoid strains to mucosal epithelial surfaces (McEachran and Irvin, 1985; Ramphal and Pier, 1985; Ramphal *et al.*, 1987). The mucoid character is chromosomally encoded and mucoid cultures arise *in vitro* from parent nonmucoid forms. This mucoidy is characteristic of strains isolated from the chronically infected lungs of CF patients (60-90%), chronic infection in bronchiectasis and in urinary tract infection, but is rare in other conditions such as in asthma patients (Pitt, 1986). Alginates have been shown to be immunogenic in humans (Pier, 1983; Pedersen *et al.*, 1989). It has been reported that the resistance to *P. aeruginosa* in older, relatively healthy, CF patients was thought to be due to the presence of opsonic killing antibodies specific to alginate (Pier *et al.*, 1987). Studies are underway to develop this alginate vaccine to increase its immunogenicity (Pier, 1989), and a MEP-Pseudomonas toxin A conjugate vaccine has been prepared and is in the early stages of human trials (Sadoff, 1989). However, a recent report by Pedersen *et al.* (1990) demonstrated that early formation of IgA and IgG antibodies to *P. aeruginosa* alginate did not prevent development of chronic infection in CF patients. This suggested that such an approach may not block adherence of mucoid *P. aeruginosa* to mucosal surfaces.

It is also thought that the appearance of mucoid morphology occurs when the expression of alginate genes in nonmucoid strains are switched on after colonization. The term for this switch is known as alginate conversion. The alginate production phenotype is unstable and mucoid strains routinely convert back to the typical nonmucoid form in the laboratory. The exact mechanism is not fully understood, but it is known that a number of genes (*algS*, *algT*, *algR*, *algB* and *algG*) are involved (Ohman and Chitnis, 1989). Thus, the initial colonizers are believed to be the nonmucoid strains (Dogget, 1969; Hoiby, 1974; George, 1987) and the adherence of these strains is mediated by polar filamentous appendages called pili (Doig *et al.*, 1988; Ramphal *et al.*, 1987; Woods *et al.*, 1980a). Early allusions to the possibility of a pilus-based vaccine were made by Woods *et al.* (1980a), who demonstrated that anti-pilus antiserum was able to block adherence of *P.*

aeruginosa to buccal epithelial cells. Inhibition of nonmucoid but not mucoid strains by anti-pilus antibodies has also been shown in tracheal cells (Ramphal *et al.*, 1987). As is evident from Table III.2, the concept of a pilus-based vaccine has not been explored and it is our intention to examine this possibility more closely.

F. Synthetic peptide vaccines

Before moving into a discussion on the *Pseudomonas* pilus, a brief discussion of the application of peptides as immunogens would be appropriate. The immune system elicits a limited response to intact proteins. Previous studies led to two main conclusions concerning the antigenic determinants of proteins. Firstly, immunization with the native protein gives rise to antibody reactivity which is confined to only a few regions of the molecule; secondly, the antigenic determinants are dependent on the tertiary conformation of the region. The use of artificial peptide antigens dates back to the work of Anderer (1963) with a hexapeptide antigen from tobacco mosaic virus (TMV). Immunization of rabbits with this BSA-peptide conjugate gave rise to an antiserum which precipitated and neutralized TMV. Some of the developments surrounding the early use of synthetic peptides as immunogens have been summarized by Sela (1969). In the early seventies, a synthetic peptide antigen was shown to induce antibodies reactive with the native lysozyme (Arnon *et al.*, 1971). In the early 80's, Lerner and coworkers demonstrated that peptides may be used to induce antibodies which react with the native hemagglutinin protein, even though the peptides were not the part of the immunogenic portions of the protein (Green *et al.*, 1982). These experiments showed that synthetic peptides representing virtually any part of the surface of a protein can elicit antibodies which can bind to the native molecule. As pointed out by Lerner (1982), "the ability of antipeptide antibodies to react with the intact structure is independent of any particular conformation or location in the molecule", provided that part of the sequence be located on the surface of the protein. The ability to raise antipeptide antibodies of a predetermined specificity is important in providing

neutralizing antibodies against bacterial and viral invasions. The important functional regions of these microorganisms are not normally immunogenic, enabling them to evade the humoral immune defense mechanism and to colonize and infect the host. The use of synthetic peptides as immunogens would elicit antibodies directed against functional regions of the microorganisms and prevent them from invading the body. Concurrent with the advancement in knowledge of molecular immunology was the advent of technological progress in peptide synthesis. Solid-phase peptide synthesis was developed by Merrifield (1963) and this represented a major improvement over the more tedious solution-phase condensation procedure. The automated solid-phase procedure simplifies and accelerates the synthesis of peptides. Small peptides can now be synthesized in a day by state-of-the-art peptide synthesizers. Analogs of a peptide sequence ten to twenty residues long with single amino acid residue substitutions can be prepared simultaneously using the "Teabag" approach (Houghten, 1985; Houghten *et al.*, 1986). Syntheses of short peptides, shifting one amino acid residue down the sequence of a protein, were carried out on polystyrene rods (Geysen et al., 1987). These immobilized peptides were then used to screen for antigenic determinants (Geysen *et al.*, 1987). With the advent of advanced techniques in peptide chemistry and synthesis, together with the advancement of knowledge in important areas of immunology during the past two decades, the quest for peptide-based vaccines has taken a major step into the forefront of research and development in universities, biotechnology and pharmaceutical companies.

The research and development of synthetic vaccines offers possible alternatives to attenuated or detoxified vaccines presently in use. These synthetic vaccines may also provide new prophylatic treatment to diseases where no vaccines are presently available. A number of problems associated with killed or attenuated vaccines have been discussed by Arnon (1986) and include (i) the difficulty in ascertaining whether the vaccine preparation is completely killed or attenuated and, thus, adequately non-hazardous, (ii) the difficulty in preparing sufficient material for vaccine production, and (iii) the genetic variation in viruses

or bacteria leading to changing serotypes and hence, requiring continuous development of new vaccines. Some of the approaches to new vaccines include subunit vaccines, recombinant vaccines, recombinant infectious vectors, anti-idiotype vaccines and synthetic peptide vaccines (Ada, 1986; Steward and Howard, 1987; see references therein). The potential of synthetic peptide vaccines offers a few advantages and disadvantages and these are listed in the Table I.3. At the present time, there are no peptide-based vaccines commercially available for human use although a few are being studied, including foot and mouth disease virus in cattle, poliovirus, human immunodeficiency virus and malaria for human use (see review by Steward and Howard, 1987).

In dealing with protein antigenicity, it is important to remember that antigenicity is used to describe the ability of a particular molecular structure to be recognized by B-cell (antibody-producing cell) and T-cell receptor molecules, while immunogenicity is the ability of a molecule, a peptide in this case, to induce an immune response, manifested either by antibody production or by cell-mediated immunity (Male *et al.*, 1987). Macromolecular antigens such as proteins usually express a multitude of possible antigenic determinants, or epitopes, that dictate their specificity. These antigenic determinants may consist of a linear sequence (sequential epitope), a linear peptide with sufficient tertiary structure (contiguous, conformational epitope) or different regions of a protein brought close together by protein folding (discontiguous, conformational epitope). Thus, the whole surface of a protein can be described as a continuum of antigenic determinants, but in reality only a few regions would be immunogenic because of the ability of the host immune system to respond to the whole molecule. Based on the the X-ray crystallographic data of the lysozyme-antibody complex, the surface area for interaction between protein and the antigen-combining site of the Fab fragment is of the order of 20 x 25 Å, leading to suggestions that protein epitopes are likely to be discontiguous and that crossreacting peptides mimic only the 'primary' interaction site (Barlow *et al.*, 1986). Hence, many

TABLE I.3. The advantages and disadvantages of Peptide Vaccines

Advantages of Peptides Vaccines

1. Chemically defined
2. Stable indefinitely, no need of refrigerated transportation
3. Problems associated with attenuated vaccines or killed vaccines are eliminated, e.g.,reversion of attenuated vaccines and incomplete inactivation of killed vaccines
4. Large scale processing is not required
5. Designed to stimulate only the appropriate immune response
6. Provides opportunities for using delayed release mechanism
7. Contamination of agents such as human immunodeficiency virus, heptatitis virus, etc., will not be present
8. Absence of other components that could cause an undesirable immunological response

Disadvantages of Peptide Vaccines

1. Need for a carrier
2. Need for an adjuvant
3. Peptide mimics of conformational epitopes are difficult
4. Possible weak reactivity of antipeptide antibodies with the native molecule

investigators have been successful in raising antisera against short peptides that recognize the native protein. Searches made for protein determinants where antipeptide antisera crossreacted with native proteins have shown that these antigenic peptides often correspond to parts of proteins which fall predominantly in the loops and/or protruding regions (Barlow *et al.*, 1986). This explains the rationale for investigators using hydrophilicity, accessibility and mobility to predict parts of a polypeptide which provide the most likely antigenic peptides. The ability of the synthetic peptide to generate antibodies which crossreact with native proteins depends entirely on the probability that the free peptide mimics the conformation of that identical sequence in the protein.

The fact that antibodies raised against peptides which are in a highly disordered state can crossreact with proteins which are in a highly ordered state has been termed as an 'ordered-disordered paradox' and has been reviewed by Dyson *et al.* (1988). This paradox is not seen in anti-protein antibodies binding to peptides where one can envisage an unfolded peptide binding to the antibody by a simple induced-fit mechanism. The binding of an antipeptide antibody to a native protein may be due to the deformation of the protein upon binding to the antibody to fit into the antigen-combining site or alternatively, may be due to the antipeptide antibody being raised to a peptide conformation that approximated that of the protein. A third possibility is that the conformation of the peptide on the carrier molecule or on the B-cell receptor is made similar to that of the peptide sequence in the native protein by a nonspecific mechanism, stabilizing the folding in a protein environment.

Although many epitopes may be expressed by a protein, only a limited number of these may provide neutralizing immunity. The relevant epitopes, which can be identified by several procedures, have been discussed by Arnon (1986). One of these procedures involves the fragmentation of the native protein and screening of the resultant fragments for immunologically active components that can bind to antibodies and can interfere with their interaction with the intact antigen. The employment of monoclonal antibodies with neutralizing activity for identifying relevant antigenic sites is a second alternative.

Potentially relevant epitopes may also be obtained from three dimensional structural models of proteins derived from X-ray crystallographic data; however this approach is limited due to the difficulties involved in forming good diffractable protein crystals. The epitopes of a protein can also be screened by systemically synthesizing all the heptapeptide units in its sequence and measuring their reactivity with antibodies against the native protein (Geysen *et al.*, 1984). An extension of this procedure which applies to neutralizing monoclonal antibodies is to substitute systematically, with all 20 naturally occurring amino acids, each residue of a peptide sequence with the highest affinity (epitope) to generate what has been termed a mimetope (Geysen *et al.*, 1985). Such a peptide bears little or no direct relationship with the original epitope, but it mimics its ability to bind specifically to the antibody. It would be interesting to see if mimetopes can generate crossreacting antibodies which have neutralizing activities.

G. *Pseudomonas* pilus: biosynthesis, structure and assembly

The occurrence of surface appendages similar to pili (fimbriae) on *Pseudomonas* species was noted by Houwink and Iterson (1950) and the visualization of pili on *P. aeruginosa* by electron microscopy was reported by Weiss (1971). The terms pilus and fimbria are used synonymously in this thesis. A pilus is different from a flagellum in that it does not have the characteristic sinusoidal longitudinal waviness of a flagellum, it is thinner in diameter, and is normally more numerous per bacterium. The *Pseudomonas* pili, classed as NMePhe pili produce a twitching motion (Henrichsen, 1972) and act as receptors for bacteriophages (Bradley, 1972). A pilus has an average length of 2500 nm. It consists of a polymer of a single subunit called pilin of molecular weight varying between 13- to 17-kilodaltons, depending on the strains from which the pilins are found. Folkard *et al.*(1981) have used X-ray diffraction to obtain structural information of pili from *P. aeruginosa* strains PAK and PAO. These filamentous structures consist of hollow cylinders of 5.2-nm diameter with a central channel of 1.2 nm. The pilin subunits are arranged in a helical

fashion with five subunits per turn (Watts *et al.*, 1983). The X-ray crystal structure of the *P. aeruginosa* pilin subunit has not been obtained. However, in a recent report by Parge *et al.* (1990), the crystallization of the NMePhe pilin subunit from *N. gonorrhoeae* was reported. Analysis of the data is underway to determine the structure of this pilin. The methodology developed by these workers may aid in the crystallization of the *Pseudomonas* pilin. Such studies are presently being pursued in the laboratory of Dr. Randy Read (Department of Medical Microbiology and Infectious Disease, University of Alberta).

The pilin gene of *P. aeruginosa* has been cloned and sequenced (Pasloske *et al.*, 1985; Sastry *et al.*, 1985a; Pasloske *et al.*, 1988). A single copy of the pilin gene is located chromosomally (Pasloske *et al.*, 1985). From the sequencing data of Paranchych and coworkers (Paranchych *et al.*, 1990), the pilins of *P. aeruginosa* are found to vary in their amino acid sequences (Figure I.1) although the extent of this polymorphism is not yet known. These workers have identified at least six unique pilin types based on nucleotide sequencing information and these pilin genes are designated PAK, PAO, CD, P1, K122-4, and KB7. The N-terminal region (residues 1-30) consists of very hydrophobic amino acid residues and is conserved in all strains. There are two moderately conserved regions, residues 31-55 and residues 105 to the C-terminus. Studies carried out by Watts *et al.* (1983) showed that the tyrosine residues at positions 22 and 27 were buried, suggesting that this region is involved in subunit-subunit interactions. The two cysteine residues at the C-terminal region are highly conserved in all pilins sequenced. In PAK and PAO, studies (Sastry *et al.*, 1985a; Sastry *et al.*, 1985b) have shown that these residues form a disulfide bond. The K122-4 pilin differs from the others in that it has an additional two cysteine residues in the central portion of the protein. It is also the longest of the NMePhe pilins in *P. aeruginosa* and it has a seven-residue leader peptide instead of six residues. The P1 pilin is unique in that it has the longest putative C-terminal disulfide loop. There are 17 residues in the loop instead of 12 residues.

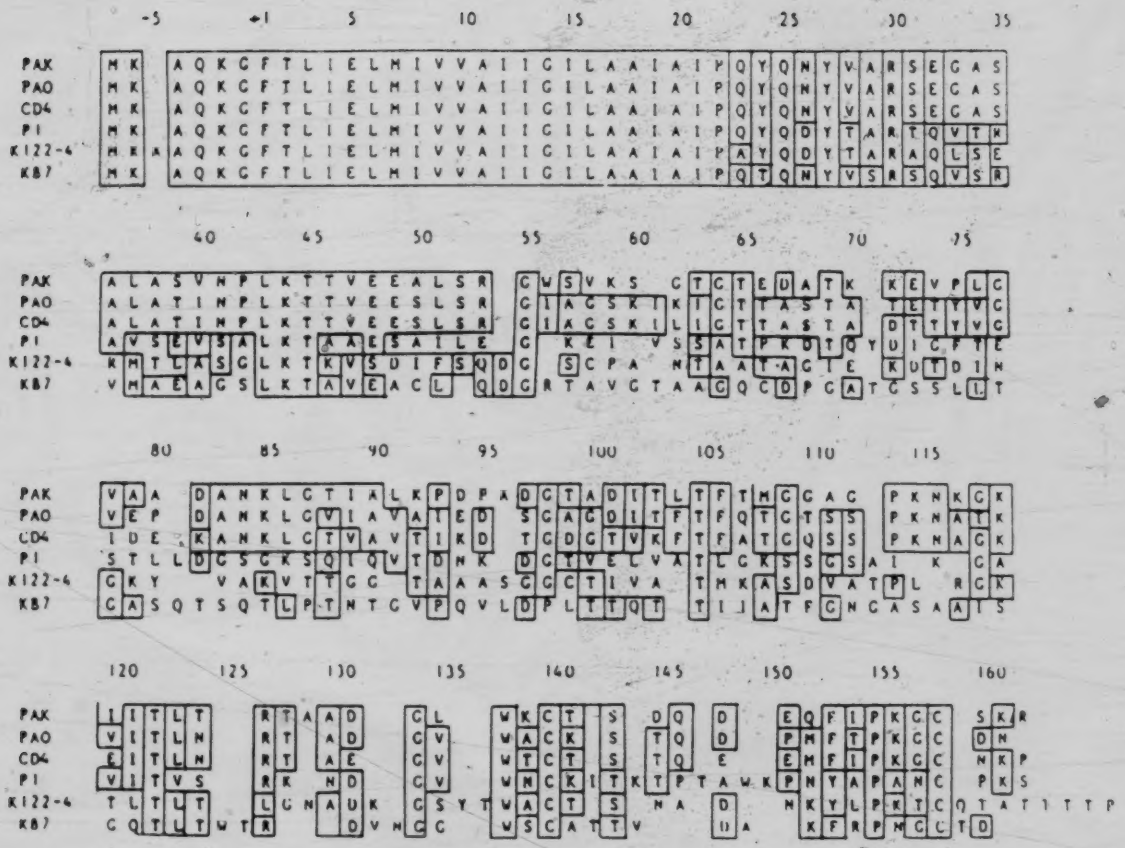


Figure I.1. Amino sequences of pilins from six strains of *P. aeruginosa* taken from Paranchych *et al.* (1990).

The expression of the pilin gene can be turned on and off but the precise control mechanism is not known. The promoter for the pilin gene has been identified by Johnson *et al.*(1986) and this promoter region is similar to a class of promoters recognized by RNA polymerase with an alternative sigma factor called RpoN (NtrA or GlnF). This RpoN sigma factor is required for transcription of the pilin gene (Ishimoto and Lory, 1989). Insertional RpoN$^-$ mutants were defective in synthesis of some enzymes involved in nitrogen assimilation such as glutamine synthetase and urease. These mutants also failed to synthesize pilin, confirming that the structural pilin gene is also under the *rpoN*-controlled global regulating network. A number of genes responsible for expression of virulence factors may also be under *rpoN* control, e.g., the flagellin gene (Lory, 1989). This would suggest that the genes specifying attachment and motility appear to be coordinately regulated (the flagellum functions as a motility organelle). RpoN-regulated genes usually respond to environmental stimuli and require one of more transcriptional regulatory polypeptides. NtrA-dependent promoters in enteric or nitrogen fixing organisms are regulated by the gene products of either *ntrC* (NtrC) or *nifA* (NifA) by binding to sequences upstream of the NtrA-dependent promoter. Such a NifA-binding sequence (115 bp) has been putatively located by Pasloske *et al.* (1988). The deletion of a 48 bp sequence from this putative recognition site resulted in a decreased level of pilin production, suggesting that the upstream region provided positive regulation of pilin gene expression (Pasloske *et al.*, 1989).

Unlike other extracellularly transported proteins of *P. aeruginosa* such as OMP-F, ETA and azurin, which have signal sequences of 15-30 residues, the pilin molecule is synthesized as a propilin with only a six- to seven-residue signal peptide which designates transport across the membrane. This has led a number of workers to postulate the possibility of a different export system for *P aeruginosa* pilin (Johnson and Lory, 1986; Paranchych *et al.*, 1990). The mature protein has about 144 amino acid residues. The N-terminus has a characteristic methylated phenyalanine (Frost *et al.*, 1978). This type of

pilus is often referred to as an NMePhe pilus (Paranchych and Frost, 1988). Pasloske and Paranchych (1988) observed that a glu to lys substitution at position 5 resulted in a processed pilin molecule with an unmethylated phenylalanine which failed to assemble into a pilus. These data suggested that the processing of the leader sequence and the methylating step are separate events. A gene designated as *pilD* has been identified by Nunn *et al.* (1990) and is located adjacent to the pilin gene and may act as the leader peptidase. When this mutated PAK pilin gene was expressed in a multipiliated PAO strain (DB2), a heteropolymeric pilus with a corkscrew-like morphology was observed (Pasloske *et al.*, 1989). The mutant pilin subunits failed to form homopolymeric filaments. When wild-type PAK and PAO pilin subunits were expressed in the same bacterial strain, the pilin subunits assembled into straight filamentous homopolymeric pili containing one or the other type of subunit but not both. Earlier studies by Watts *et al.* (1982) revealed pools of pilin in the outer and inner membrane of *P. aeruginosa*. It was not shown whether the two pools of pilin were the mature NMePhe pilin or the unprocessed form. However, one would expect the outer membrane pool to be in the processed form. The assembly of the mature pilin subunits into a functional pilus is believed to occur at the outer membrane or at the Bayer junctions, where the outer and inner membrane are fused. The number of accessory gene products required for pilus assembly in *P. aeruginosa* has not yet been established.

If the assembly of the NMePhe pilus is anything like that of the type 1 or Pap pilus, a complicated set of accessory gene products would be involved in the assembly process. However, the NMePhe pili are quite different from those found in organisms expressing type 1 or Pap pili. Thus, some differences in the posttranslational processing and organelle assembly would be expected. As mentioned earlier, the processing of the short leader peptide in the *Pseudomonas* propilin into an N-methylated mature pilin molecule and its transport for assembly would occur by different mechanisms. The RpoN-dependent activation of the pilin gene transcription may sense the fluctuation in either the rate of

assembly or the level of pilin subunits (Johnson and Lory, 1987). The assembly of pilin subunits seems to be a very specific process in that the one type of pilin only associates with its own type to form homopolymeric pili, even when two different pilin types (PAK and PAO) are expressed in the same cell (Pasloske *et al.*, 1989). The same or similar machinery seems to be employed in the assembly of NMePhe pili in other species. Recombinant *P. aeruginosa* containing the *B. nodosus* pilin gene was able to assemble a Bacteriodes pilus which was structurally and antigenically indistinguisable from that produced by *B. nodosus* (Mattick *et al.*, 1987). A similar observation was made with *M. bovis* NMePhe pili expressed in *P. aeruginosa* (Elleman and Peterson, 1987). However, in the report by Pasloske *et al.* (1989), mutant pilin subunits with a glu to lys substitution at position 5 at the highly conserved N-terminal region were able to be incorporated with PAO wild-type subunits to form heterologous pili with unusual corkscrew-like morphology instead of the normal straight filamentous strands. Since both pilin types were derived from the same species and hence, have very similar features, it would have been possible for the two pilin types to form this unusual morphology. However, a recent report by Beard *et al.* (1990) showed that it was possible to obtain the expression of heterologous pili containing pilin subunits from different species (*M. bovis* and *P. aeruginosa*). The NMePhe pilins share a highly conserved N-terminal of about 30 residues (Paranchych, 1989). This region is believed to be involved in subunit-subunit interactions (Watts *et al.*, 1983). The occurrence of heterologous mosaic pili may then be explained on the basis of reversibility and relative stability of subunit-subunit interactions in the filamentous strand (Beard *et al.*, 1990). The pilus can undergo retraction-protraction which would involve the assembly and disassembly of the structural pilin subunits. Watts *et al.* (1982) have reported membrane associated pilin pools on both the outer and inner membrane. Thus, Beard *et al.* (1990) proposed " that site-of-entry reversibility of subunit assembly could account for the different types of pili produced in cells expressing more than one type of subunit, if interactions between homologous subunits are relatively stable, whereas

interactions between heterologous subunits are only stable if the subunits are sufficiently similar in structure. Hence, stable interaction would favor the forward equilibrium and assembly of similar subunits at the expense of unstable interactions between dissimilar subunits ". This model would explain the existence of mosaics of *Pseudomonas* and *Moraxella* pilin subunits because of the relatively stable stacking between the subunits which share similarities in their primary and secondary structures. This interchangeability of the NMePhe pilin may not be absolute in that the expression of the gonococcal pili in *P. aeruginosa* was not successful (Beard *et al.*, 1990). The NMePhe pilin subunits are synthesized in the cytoplasm and would have to be transported across the periplasmic space to the outer membrane of the cell to be assembled.

The number of accessory genes required for the assembly of the *Pseudomonas* pilus is still unclear. Three of the genes *pilB*, *pilC* and *pilD*, were recently reported by Nunn *et al.* (1990) to be necessary for biogenesis of the pilus. The gene products consist of polypeptides of 566, 289 to 366, and 252 to 290 amino acids. Although these polypeptides have not been characterized, these authors suspected, from hydropathy plots, that PilC and PilD are integral membrane proteins, while PilB is cytoplasmic. In the Pap pilus assembly process, an accesory protein called PapD, acts as a chaperone to transport the structural pilin subunit to the outer membrane for assembly (Lindberg *et al.*, 1989). The crystal structure of this protein has also been reported (Holmgren and Branden, 1989). Such a protein has not been identified in *Pseudomonas*. Unlike the NMePhe pilus of *P. aeruginosa*, the Pap, type 1 and S pili of *E. coli*, consist of more than one type of subunit (Lindberg *et al.*, 1984; Maurer and Orndoff, 1987; Schmoll *et al.*, 1989). Organized at the tip of the Pap pilus, are proteins different from the structural pilin subunit. These tip proteins are the adhesins, molecules containing the receptor binding function. The Pap pilin operon consists of more than 10 genes with gene products serving different functions. The type 1 pilus also has a number or minor proteins associated with it (Abraham *et al.*, 1987; Maurer and Orndoff, 1987). In the type 1 pilus, the adhesin called FimH is

associated with two other proteins, FimF and FimG (Klemm and Christansen, 1987). From immunoelectron microscopy studies, the FimH protein was shown to be laterally located along the pilus shaft (Krogfelt *et al.*, 1990). The S pilus of *E. coli* also has at least four genes in its operon (Schmoll *et al.*, 1989). A model of the assembly of the various subunits into a functional pilus has been presented by Hultgren *et al.* (1989).

H. The role of pili in adherence

The first stage in the establishment of a bacterial infection is believed to be the adherence and colonization on the mucosal surfaces of the host. This represents the initiation of infection. Johanson *et al.* (1972) reported that colonization of the respiratory tract with gram-negative bacilli apparently played a major role in the pathogenesis of nosocomial infection. In their study, they found that 22 of 99 patients (23%) who were colonized developed such infections, compared with 4 of 118 (3%) patients where colonization was not observed. Subsequent studies by Johanson *et al.* (1979) showed that colonization of the respiratory tract with gram-negative bacilli was accompanied by adherence of the colonizing species to epithelial cells of the upper respiratory tract. *In vitro* studies showed that more gram-negative bacilli (*P.aeruginosa* and *K. pnuemoniae*) adhered to colonized cells than noncolonized cells. Gram-negative bacillary colonization of the upper respiratory tract was associated with increased adherence of *P. aeruginosa* to buccal epithelial cells (Johanson *et al.*, 1980). As stated by Beachey (1981), it is generally true that bacterial adherence is a prerequisite to bacterial infectivity. In his review of a number of organisms (*Neiserria, Escherichia, Streptococcus, Salmonella, Proteus, Bordetella*), he found that there was a parallel relationship between adherence of bacteria to epithelial cells in vitro and bacterial infectivity *in vivo* with the exception of Salmonella where the nonfimbriated strain also showed moderate infectivity. Nonadherent enterotoxigenic *E. coli* strains were also found to be shed in stools much faster than adherent strains (Satterwhite *et al.*, 1978). In pigs lacking receptors for the K88 fimbriae of *E.coli* on their

mucosal epithelial brush borders, it was found that they were genetically immune to these infections (Sellwood *et al.*, 1975).

As mentioned earlier, two adhesins have been reported. The epithelial tissues to which *P. aeruginosa* adhere include hamster tracheal epithelium (Baker and Marcus, 1982), human respiratory epithelial cells (Doig *et al.*, 1988; Ramphal and Pyle, 1983), human buccal epithelial cells (McEachran and Irvin, 1985; Woods *et al.*, 1980a) and human corneal epithelium (Reichert *et al.*, 1983). Pili-mediated adherence in *P. aeruginosa* was first reported by Woods *et al.* (1980a). Pilus-mediated adherence has previously been demonstrated in gonococci infections on mucosal surface of genitourinary tract (Swanson, 1973). The establishment of gonococcal infection was correlated with the presence of pili on the surface of virulent organisms. Similar observations have been made in *E coli* infection in piglets where the K88 fimbriae were implicated in adherence (Jones and Rutter, 1972). In *M. bovis*, fimbriation showed a relation with keratoconjunctivitis in cattle (Pedersen *et al.*, 1972). Woods *et al.* (1980a) reported that purified pili from a *P. aeruginosa* strain, DG1, caused a significant decrease in adherence of *P. aeruginosa* to human buccal epithelial cells. In addition, they also found that antisera to purified pili prepared from strain, DG1, decreased adherence of DG1 to buccal epithelial cells but had no effect on strain, PK. Ramphal *et al.* (1984) reported pili-mediated adherence of nonmucoid *P. aeruginosa* strains to injured tracheal epithelium. They also found that both homologous and heterologous pili inhibited a nonmucoid strain from adhering to the tracheal epithelium. In addition, pili also inhibited two nonmucoid strains derived from their mucoid variants but did not inhibit the mucoid strains from adhering to tracheal epithelium. The data suggested that the pili-mediated adherence may have a common receptor or closely positioned receptor sites and that nonmucoid and mucoid strains have different receptors. Sato and Okinaga (1987) showed that pili-mediated the adherence of *P. aeruginosa* to mouse epidermal cells and that nonpiliated mutants were found to be less virulent than the isogenic piliated strains. These authors reported that the 50% lethal dose

of piliated mutants was at least 10 times lower than that of nonpiliated mutants. Some direct evidence of *P. aeruginosa* pili being an important virulence factor has been presented by Paranchych and coworkers (Paranchych and Frost, 1988). In mice that were immunosuppressed, the LD50 values for a Pil^- strain of PAO were 100-10,000 fold higher than those of an isogenic Pil^+ strain. These workers were also able to show that administration of anti-pilus IgG at the time of challenge afforded passive immunity to the mouse. The LD50 values were 10,000-fold higher than in control mice given non-immune IgG.

Woods *et al.* (1980b) reported that increased adherence of *P. aeruginosa* to buccal epithelial cells *in vitro* varied directly with the loss of fibronectin. The loss of fibronectin was also associated with an increase in salivary proteases in the CF patients. Trypsinization of normal cells also increased bacillary adherence and reduced the amount of fibronectin. Furthermore, Woods *et al.* (1981) proposed that fibronectin in cell surfaces may inhibit adherence by occupying specific sites on cells or sterically blocking the pilus from interacting with the receptors. Paranchych *et al.* (1985) have used a wide variety of synthetic di-, tri-, and tetra- saccharides found in mammalian glycoproteins and glycolipids which failed to show any significant binding activity to PAK pili. They found phosphorylase *b*, pyruvate kinase and aldolase showed high binding activities, leading the authors to propose that a polypeptide with structural similarities to these enzymes may be the receptor rather than an oligosaccharide. However, Vishwanath and Ramphal (1985) reported that receptors on injured tracheal epithelium were sensitive to periodate oxidation. Furthermore, it was found that nonmucoid *P. aeruginosa* strains were sensitive to inhibition by N-acetylneuraminic acid. Treatment of the tracheal cells with neuraminidase, but not pronase, significantly reduced adherence of these organisms. Ramphal *et al.* (1987) also reported that the binding of nonmucoid *P. aeruginosa* strains to isolated human tracheobronchial mucins was inhibited by anti-pilus antiserum, suggesting that at least a part of the receptor for the pili-mediated adherence was comprised of carbohydrates.

McEachran and Irvin (1985) have demonstrated that there are two classes of receptors: a high affinity-low copy number site for the pili binding site and a high copy number-low affinity site for the alginate adhesin. The high affinity site for pili was inhibited by N-acetylneuraminic acid and D-arabinose, whereas the low affinity site for the alginate adhesin was inhibited by D-fucose which lacks a hydroxyl group on the C-6 position of the sugar ring. More recently, Doig *et al.* (1990) and Irvin *et al.* (1989) were able to show the presence of glycoproteins in membranes of buccal epithelial cells which demonstrated pili binding activities.

There may be other adhesins involved in the attachment of *P. aeruginosa* to epithelial cells. Mutants of *P. aeruginosa* strain PAK which do not express pili on their surface have been constructed. These mutants were still able to adhere to human pulmonary carcinoma A549 cell line. Although the level of binding was less than 25% of that of the wild-type, it did represent a significant level of adherence (Lory, 1989). This has led to the suggestion that more than a single adhesin is involved in bacterial attachment to a mucosal surface. The expression of other Pseudomonas genes such as proteases and flagellin were found to be required to promote efficient binding to epithelial cell monolayers (Saiman *et al.*, 1990).

If bacterial adherence is such an important stage in the infection process, it would seem that the blockage of this step could theoretically eliminate or at least reduce the risk of infection. Indeed, one of the goals of the elucidation of the mechanism of adherence would be the eventual development of measures to prevent adhesion of harmful bacteria to mucosal surfaces before the organisms can produce tissue damage (Beachey, 1981). In Beachey's review, he mentioned three methods in which anti-adhesives may work:

i) the application of purified adhesins or analogs as competitive inhibitors of bacterial adherence

ii) the admininstration of drugs that specifically suppress the formation or expression of bacterial adhesins, e.g., sublethal dose of antibiotics

iii) the development of adhesin vaccines to induce the formation of local antibodies that could coat the organisms and thereby prevent adhesion.

Intravesicular administration of mannose to mice which had been challenged with an adherent *E. coli* strain reduced colonization by the bacteria to 20% *versus* 70% in control mice (Aronson *et al.*, 1979). O'Hanley *et al.*, (1985) reported that analogs of gal-gal disaccharide inhibited the hemagglutination by Pap pili. Beachey and coworkers (Alkan *et al.*, 1978; Ofek *et al.*, 1979; Eisenstein *et al.*, 1979) have demonstrated that doses of antibiotics caused the loss of *S. pyogenes* adherence to human oral epithelial cells (penicillin) and also *E. coli* adherence (by streptomycin). The authors proposed that the reason for the loss of adherence may be due to loss of fimbriae formation on these organisms.

The most effective and long lasting protection against adherence would be the employment of the host immune defences. The purified adhesin or its analog would serve as an immunogen inducing antibodies that can reach mucosal surfaces, affording local immunity to bacterial infections. An example of this anti-adhesive approach has been demonstrated with the K88, K99 and K987 fimbriae where these purified vaccines gave rise to protection against diarrheagenic *E. coli* in pigs (Rutter and Jones, 1973; Isaacson *et al.*, 1980). Adhesin vaccines against *N. gonorrhoea* are also being developed (Tramont *et al.*, 1980; Brinton *et al.*, 1980).

I. Aims of the project

At present, there are no effective vaccines for *P. aeruginosa* infection using pili as the immunogen. The effectiveness of pili as immunixing agents has been demonstrated by the examples of K88, K99, and K987 fimbriae preventing adhesion of *E. coli* to bovine

and porcine epithelial cells. *Bacteroidus nodosus* pili have also been used to vaccinate sheep (Egerton *et al.*, 1987). As mentioned earlier, Paranchych and coworkers have demonstrated passive immunity with anti-pilus antibodies in mice challenged with live *P. aeruginosa* organisms. However, the *P. aeruginosa* pili could display as many as 10 serotypes and the ability of an anti-pilus antibody being effective against all strains is limited. So there is a need to find some conserved region which can be use to generate neutralizing antibodies effective against a wide spectrum of the *P. aeruginosa* strains. To get neutralizing antibodies, we need to delineate the region of the pilin molecule which is important to bacterial adherence. The question of the immunogenicity of this region would be important in the development of an effective vaccine that would give high titer neutralizing antibodies. Thus, the aims of the project are as follows:

i) to map the surface region of *P. aeruginosa* pilin - synthetic peptides corresponding to the sequence of pilin would be used to raise antibodies. The surface location of the peptide region would be assessed by the ability of the antipeptide antibodies to interact with the native protein.

ii) to delineate the region/s conferring receptor-binding activities by assessing the abilities of the antibodies to block pili and bacterial binding to epithelial cells.

iii) to determine the antigenicity and immunogenicity of the region of pilin conferring receptor binding activity. This would allow the assessment of the ability of the peptide region to induce high titer antibody production. The abilities of the peptides to be bound by antibodies raised against the whole pilus would also have to be determined. These studies would allow the assessment of the suitability of the receptor-binding region of pilin as a vaccine candidate against *P. aeruginosa* infections.

Chapter II. Materials and Methods

A. Selection of peptides - Surface prediction

The selection of peptides to be synthesized was based on surface predictions using a surface prediction algorithm, Surfaceplot, available from S.P.I. Synthetic Peptides Inc., University of Alberta, Edmonton, Canada. The prediction algorithm, developed by Parker *et al.* (1986), incorporated three commonly used prediction parameters: HPLC hydrophilicity parameters (Parker *et al.*, 1986), accessibility parameters (Janin, 1979) and flexibility or mobility parameters obtained from the temperature factors of α carbon atoms in the X-ray structure of proteins (Karplus and Schulz, 1985). The regions on the surface tend to be more flexible than regions that are packed in the interior of protein molecules. Hence, their flexibility parameters were included in the Surfaceplot program. The HPLC hydrophilicity coefficients were experimentally derived from the retention times of 20 model synthetic peptides with the sequence Ac-Gly-X-X-$(Leu)_3$-$(Lys)_2$-amide, where X was substituted with the 20 amino acids found in proteins. The accessibility parameters for each of the 20 amino acids, which were derived from X-ray crystal structures of proteins, represent the ratio of the mole fraction of the amino acid residues found buried to the accessible mole fraction. The Surfaceplot program was run on an IBM AT-Personal Computer. In this program, the surface profile for a protein was determined by the summation of the parameters for each residue of a seven-residue segment and assigning this sum to the fourth residue. This procedure was repeated by shifting the segment one residue at a time from the N- to the C- terminus. Each scaled plot was superimposed on the other and the maximum value of each residue was used to give the final composite profile value. In the selection of the peptides likely to be on the surface of the protein, the program empirically assigned profile values greater than 25% above the average hydrophilicity to be surface sites. This 25% value was calculated as 25% of the difference between the maximum value in the plot and the average surface profile value. The combination of the

three parameters provided an improvement (over any single parameter alone) in the prediction of antigenic sites. A study by Strynadka *et al.* (1988) clearly demonstrated the importance of a reliable prediction program. Their results revealed that synthetic peptides to seven surface sites on the glycoprotein D of Herpes Simplex virus, not predicted by the commonly used hydropathy parameters of Hopp and Woods (1981) but predicted by Surfaceplot, gave rise to antipeptide antisera that bound to the HSV-1 virions and three of the seven peptides provided neutralizing antipeptide antisera. Thus, Surfaceplot was used in this study to map the surface regions of the PAK and PAO pilin molecules.

B. Peptide synthesis

The key to peptide synthesis is to mimic the chemistry of peptide bond formation of the naturally occurring biological translational machinery. Peptide bond formation is catalyzed by a peptidyl transferase. The amino acid to be added to a peptide chain is activated through esterification of the α-carboxyl group by amino-acyl specific tRNA. This makes the α-carbonyl group susceptible to a nucleophilic attack by the free α-amino group on the peptide chain. A summary of this reaction is illustrated by Stryer (1988). Another approach to peptide synthesis would be the enzymatic route, where the equilibria of the hydrolytic enzymes are modified artificially such that the peptide bond is ligated instead of hydrolyzed. The approach which was taken in the course of this thesis research involved the chemical formation of the peptide bond. The method employed was the solid-phase peptide synthesis (SPPS) approach developed by Merrifield (1963) who was awarded the 1984 Nobel Prize in Chemistry for this simple yet elegant innovation. In this method, an insoluble support consisting of a styrene-divinylbenzene polymer serves as an anchor for the elongating peptide chain which is attached to the resin support via its C-terminus by an ether linkage. In this manner, excess reagents, byproducts from the reactions and solvents can be removed by washing the peptidyl polymeric resins with appropriately selected solvents.

There are different chemistries which can be applied to SPPS with two of the common ones employing either the tertiary butyloxycarbonyl (Boc) or the 9-fluorenylmethyl oxycarbonyl (Fmoc) chemistry. The different chemistries relate to the removal of the N^{α}-protecting groups during synthesis. The Boc group is removed by a rather harsh acid treatment. The Fmoc chemistry proposed by Atherton *et al.* (1978) uses a base-labile protecting group for the protection of the N^{α}-amino group. In the Fmoc chemistry, the peptide is anchored to the support by an acid-labile linkage and the side-chain butyl esters and ethers protecting groups are also acid labile. In this manner, the peptide chain along with the side-chain protecting groups are easily removed by acid treatment (95% TFA). The big drawback in using the Fmoc chemistry is the high cost of the Fmoc amino acids. Although compared to the Fmoc approach, the Boc chemistry involves harsher conditions during deprotection of the N^{α}-amino group and also during cleavage of the peptide from the resin with anhydrous hydrogen flouride (HF), our laboratory has been able to achieve good syntheses over the last 15 years and have not encountered any major problems with this chemistry. The peptide synthesis described utilized Boc chemistry. Some excellent reviews and practical books on SPPS are available (Barany and Merrifield, 1979; Bodanszky, 1988; Kent, 1988; Steward and Young, 1984).

The basic scheme of SPPS involves esterification of the first amino acid to the solid support. The addition of subsequent amino acids from the C → N direction involves three processes. The first of these is the deprotection step, where the N^{α}-tertiary butyloxycarbonyl (Boc) -protecting group of the amino acid residues on the chain is removed by acidolysis using trifluoroacetic acid (TFA). The second step involves the neutralization of the amino group with a base such as diisopropylethylamine (DIEA). The final step is the coupling process, where the peptidyl resin is extended by the stepwise addition of the next activated Boc-amino acid to the peptide sequence. After the coupling process is completed, the whole process of deprotection, neutralization and coupling is repeated with the next Boc-amino acid. In the event of an incomplete coupling, a second

cycle of coupling of the same amino acid is repeated. In this case, only the neutralization and coupling steps are carried out. The extent of the coupling is monitored by picric acid (Hodges and Merrifield, 1975) or by a ninhydrin test (Kaiser *et al.*, 1969).

1. *Boc-protected amino acids and their side-chain protecting group*

The N^{α}-amino group and the functional groups on the side-chains of amino acids are reactive species which must be protected with groups which can be removed after the synthesis is completed. In the Boc chemistry, the N^{α}-protecting group is tertiary butyloxycarbonyl (Boc). The side-chain protecting groups are usually benzyl esters and ethers. The Boc protected amino acids were obtained from Institute Armand Frappier (Laval, Quebec, Canada) or from Peptide Research Foundation (Osaka, Japan). The Boc-protected amino acids together with their respective side-chain protecting groups (in parentheses) that were used in the synthesis of peptides are listed below:

ala	lys (2Cl-Z)
arg (tosyl)	nle
asn	phe
asp (β-OBzl)	pro
cys (S-MeOBzl)	ser (OBzl)
glu (γ-OBzl)	thr (OBzl)
gln	trp (formyl)
gly	tyr (OBzl)
ile	val
leu	

2. *Addition of the first amino acid to the resin*

In syntheses of peptides with a free carboxyl group, a 1% crosslinked chloromethyl Merrifield resin (Pierce, 0.9 meq/gram substitution) was used. In more recent syntheses, a more stable resin, phenylacetamidomethyl (PAM) developed by Mitchell *et al.* (1978) was used. In syntheses of peptides with an amidated C-terminal, 1% crosslinked benzhydrylamine resin (Pietta and Marshall, 1970) with a resin substitution of 0.6-0.9 meq/gram (IAF, Laval) was used. In synthesis of peptides with a free carboxyl group at the C-terminus (PAK 128-144), the carboxyl terminal lysine residue was first coupled to the Merrifield resin via a cesium salt of the Boc-Lysine (2 chlorobenzoxycarbonyl). The salt was prepared using the method of Gisin (1973). Boc Lys (2Cl-Z) (5.0 g) was dissolved in 30 ml ethanol and diluted with approximately 10 ml of water. The pH of the solution was adjusted to 7.0 by adding 2.6 g of crystalline cesium hydrogen carbonate (Aldrich) assuming a 1:1.1 molar ratio of salt to Boc-amino acid. The pH should not exceed 7.0 as excess cesium bicarbonate is not removed by evaporation. This mixture was evaporated to dryness using a rotary evaporator and washed with benzene (3 x 10 ml), resulting in the appearance of white amorphous solid. The precipitate was dried overnight over P_2O_5 in an evacuated dessicator to provide 6.0 g cesium salt of Boc-Lys (2Cl-Z).

The Merrifield resin (4.7g) was esterified with a 2 molar excess of the cesium salt of the Boc-amino acid (4.6g). The mixture was brought up in 35 ml of dimethylformamide (DMF) (6-8 ml DMF/g resin) and stirred for 20 h in a water bath at 50 °C. The mixture was then filtered and washed successively (3 x 100ml) with DMF, DMF/H_2O (9:1, v/v), water, ethanol, dichloromethane and ethanol. The Boc-Lys (2Cl-Z) resin was dried to yield 6.19 g.

The substitution of the protected amino acid on the resin can be assessed by carrying out amino acid analysis of an acid hydrolysate of a given amount of the resin. This involves hydrolysis in a 1:1 (v/v) mixture of 12 N HCl and propionic acid in an evacuated tube at 165°C for an hour (Moore, 1978; Westall *et al.*, 1972). An alternative

method was used in this study, where the substitution was determined by deprotecting the N^{α}-amino groups of the lysine residue and the amount of free amino group subsequently determined by picric acid titration (Hodges and Merrifield, 1975). The substitution of lysine on the resin approached 100% esterification (0.9 mmole/gram).

3. *Removal of the Boc-protecting group from the Boc-peptidyl resin*

The syntheses were carried out on an automated synthesizer (Beckman 990 Peptide Synthesizer; Beckman Instruments, Palo Alto, CA, USA). The basic program used on the Beckman 990 synthesizer is shown in Table II.1 and has been previously described (Parker and Hodges, 1985; Worobec *et al.* 1985). Each amino acid is coupled twice to ensure maximal incorporation of Boc-amino acid to the growing peptide chain. In order to check whether the yield of the coupling approaches 100%, a picrate monitor step (Operation VII, Table II.1) is included. Incomplete coupling often necessitates the acetylation of unreacted free amino groups (Operation VIII, Table II.1) to facilitate purification of the products after the synthesis is completed. The reaction steps for the synthesis were carried in a reaction vessel fitted with a glass sintered filter to retain the solid matrices during the washes and removal of solvents and excess reagents. The removal of the N^{α}-protecting group represents the first step in the addition of the next amino acid to the growing peptide chain. The Boc-protecting groups were removed using a 50% TFA/DCM solvent (2-min and 20-min washes). Excess reagents and the byproducts from deprotection were removed from the resin with 6 x 1min washes with DCM.

4. *Neutralization of the N^{α}-amino group*

The coupling of the next amino acid requires the N^{α}-amino group of the growing peptide chain to be in the uncharged form. Therefore, it was vital to deprotonate the amino group after the acidic treatment with TFA. The neutralization step involved two washes

Table II.1 Basic Program for the Beckman 990 Synthesizer

Operation No.	Step No.	Operation	No. of Washes	Mix Time (Min)
I		Deprotection		
	1	DCM	2	0.5
	2	Prewash: 50% TFA/DCM	1	1
	3	Deprotection: 50% TFA/DCM	1	20
	4	DCM	5	1
	5	Isopropanol	3	2
	6	DCM		
II		Neutralization		
	1	DIEA/DCM (5%)	3	2
	2	DCM	4	1
III		First Coupling		
	1	Delivery of Boc-amino acid (Manual addition of active esters or symmetric anhydrides)		5
	2	Delivery of DCC - Coupling initiated	30	
	3	DCM	1	1
	4	Isopropanel	3	5
	5	DCM	6	1
IV		Neutraliztion		
	1	DIEA/DCM (5%)	2	2
	2	DCM	6	1
VII		Second Coupling		
	1	Delivery of Boc-amino acid		5
	2	Delivery of DCC-Coupling initiated	30	
	3	DCM	1	1
	4	Isopropanol	4	1
	5	DCM	4	1

VIII		Picrate Monitor		
	1	DIEA/DCM (5%)	2	2
	2	DCM	6	1
	3	Picric acid/DCM (0.1M)	2	4
	4	DCM	8	1
	5	DIEA/DCM (5%)	2	1
	6	DIEA/DCM (5%) *	2	2
	7	DCM *	4	2
	8	DCM	2	1

* In steps 6 and 7, the solvents are collected and the picric acid is monitored spectrophotometrically at 362 nm to determine the amount of deprotected N^{α}-amino group left unreacted.

IX		Acetylation		
	1	DIEA/DCM (5%)	2	2
	2	DCM	6	1
	3	Acetic anhydride: Toulene: Pyridine (1:3:3)		30
	4	DCM	1	1
	5	Isopropanol	4	1
	6	DCM	4	4

with DIEA (2 and 10 min), after which the resin was washed with DCM (4 x 1min). In cases where the benzhydrylamine resins were used for peptides with an amidated C^{α}-terminal, the resins were washed and neutralized with DIEA prior to coupling to the first amino acid.

5. *The coupling step - Peptide bond formation*

The subsequent Boc-amino acid can be coupled to the growing peptide chain using a variety of methods which activate the carboxyl group. In SPPS, the reaction must approach 100% in order to obtain a good yield of the synthetic peptides. For an 11-residue peptide, a yield per step of 96% will only give rise to 66% of the final product. For a 21-residue peptide, this would only be 44% of the final product (Kent and Lewis, 1985). A number of unwanted side products would result due to incomplete coupling of amino acids at each position of the peptide sequence.

The first of these coupling approaches involves the use of dicyclohexylcarbodiimide (Shiehan and Hess, 1955). The advantage of the carbodiimide is that the reagent can be added to the mixture of carboxyl and amine components, allowing concurrent activation and coupling to proceed. In a typical synthesis at a 0.5 mmole scale, 3 molar equivalents (1.5 mmole) of Boc-amino acid were used to achieve a 100% reaction. A slightly higher equivalent (3.3 mmole) of DCC was used. This higher equivalent takes into account some moisture in the DCC reagent. In this procedure, an intermediate, O-acyl isourea is formed upon addition of the carboxylic acid to the DCC, the imine (N=C) group provides powerful activation which leads to coupling (Bodanszky, 1988). This approach was used for activation of most of the Boc-amino acids.

The rate of coupling of Boc-threonine is generally slow. In order to speed up this reaction, a symmetric anhydride approach introduced by Hagenmaier and Frank (1972) was used. The generation of the symmetric anhydride was achieved by adding two molar equivalents of Boc-amino acid to DCC. An insoluble side-product, N, N'-dicyclohexyl

urea (DCU), resulted from this reaction. The symmetric anhydride of Boc-threonine (3 equivalents to the growing peptide chain) was filtered using a sintered glass filter or a glasswool-plugged Pasteur pipette, prior to addition to the neutralized amino group of the peptide chain. The reaction was allowed to proceed for one hour. Because only half of the symmetric anhydride molecule actually participates in the reaction, half of the boc-threonine would be liberated. A further 3.3 equivalents of DCC (dissolved in ~1ml DCM) was added to the reaction mixture to force the coupling of the liberated Boc-threonine to any free amino groups that might be present. The reaction was allowed to proceed for another 30 minutes.

A third coupling method, involving the use of an auxiliary coupling nucleophile, 1-hydroxybenzotriazole (HOBt), was introduced by Konig and Geiger (1970). In a typical synthesis at a 0.5-mmole scale, 3 equivalents of Boc-amino acid and 3.5 equivalents of HOBt were dissolved in chilled DMF (5-7ml). Then, 3 equivalents of DCC were added to this mixture and left for 30 min at room temperature. This yielded an O-acyl-1-hydroxybenzotriazole which is a powerful acylating agent. The HOBt reduces the extent of racemization of the slow reacting esters of Boc-amino acids (Konig and Geiger, 1970). It also reduces the extent of O → N acyl migration leading to N-acylurea, an unreactive byproduct (Bodanszky, 1988). These active esters were used for the coupling of Boc-glutamine to the peptide chain.

The cycle time for the first coupling on the Beckman 990 synthesizer was 2.5 hours plus another 1.5 hours for the second coupling. Some of the peptides were synthesized on the newer Applied Biosystems peptide synthesizer Model 430A. The basic outline of the program for this new state-of-the-art synthesizer is shown in Table II.2 and has been previously described (Hodges *et al.*, 1988; Kent and Lewis, 1985). The cycle time for a single coupling was one hour and another forty-five minutes for a second coupling. The Applied Biosystems synthesizer employs the symmetric anhydride in the coupling step as it provides a faster coupling rate. Unlike the Beckman 990 synthesizer where one has to

Table II.2. Basic Program for the Applied Biosystem Synthesizer 430A

Operation No.	Step No.	Operation	No. of Washes	Mix Time (Min)
I		Deprotection		
	1	33% TFA/DCM	1	1.3
	2	50% TFA/DCM	1	18.5
	3	DCM	3	1
II		Neutralization		
	1	10% DIEA/DMF (v/v)	2	1
	2	DMF	5	1
III*		Activation of Boc-amino acid (Symmetric anhydride formation) While the first two operations are in progress the synthesizer begins the preparation of the symmetric anhydride. Boc amino acids are dissolved in DCM and delivered to the Activator vessel. Half equivalent of DCC is added the Boc-amino acid to activate and form the symmetric anhydride. The solution is filtered (to remove the dicyclohexyl urea) into a concentration vessel. DCM is evaporated and replaced with DMF.		
IV		Coupling		
	1	Delivery of preformed symmetric anhydride		30
	2	DMF	3	0.75
V		Neutralization		
	1	10% DIEA/DMF	1	1
	2	DMF	3	1

VI*		Activation of Boc-amino acid (Symmetric anhydride formation) During the latter part of the first coupling step, the symmetric anhydride formation initiated and is ready for coupling immediately after the DMF washes are completed.		
VII		Second coupling		
	1	Delivery of symmetric anhydride		
	2	DMF	1	1
	3	DCM	5	1

---

* The coupling of asn, gln, arg and BBA utilizes the HOBt active ester route and the coupling is carried out in DCM. (Refer to Applied Biosystems Users' Manual for the Model 430A peptide synthesizer, pp. 6-36).

prepare the symmetric anhydride manually, the Applied Biosystems synthesizer is designed to generate the symmetric anhydride as well as HOBt active esters (for coupling of asn, gln, arg and benzoylbenzoic acid) in the activator vessel, filter away the DCU byproduct and concentrate the activated Boc-amino acid before addition to the growing peptide chain in the reaction vessel.

6. *Cleavage and extraction*

Following the completion of synthesis, the peptidyl resins were dried and the peptides were cleaved from the resin by the method of Lenard and Robinson (1967) using anhydrous hydrogen fluoride (HF). The cleavage was carried out in an evacuated system in a fumehood because of the danger of volatile HF. For one gram of peptidyl resin, 10 ml of HF and 1 ml of anisole scavenger were used for the cleavage of peptides with a benzoylbenzoyl moiety on the peptide chain. Peptides without this moiety were cleaved by further addition of 5 drops of ethanedithiol which act as a scavenger for free radicals released during the HF cleavage. The cleavage mixture was stirred for 45 min in a salted ice bath, where a temperature of -5 to -10 °C was maintained. After this time, the HF was evaporated off and the resins dried. Extraction of the peptide was carried out by first washing the resins with diethylether (BDH, Poole, England) to remove organic byproducts. The solvent was filtered off by suction through a coarse sintered glass filter. This was repeated three times with 10 ml of ether. The peptides were extracted with 3 x 10-ml of either neat TFA or 30% acetic acid. TFA was immediately evaporated off after extraction to leave behind the crude peptides. The peptide was dissolved in 75-100 ml of deionized water and freeze-dried. In the extraction with 30% acetic acid, the acetic acid solution was diluted to at least 5% (v/v) acetic acid before freeze drying. A list of the peptides which were synthesized for the project is tabulated in Table II.3.

Table II.3 List of Peptides Synthesized for this Project

PAK 22-33	Ac-P-Q-Y-Q-N-Y-V-A-R-S-E-G-NH_2
	BB-Nle-P-Q-Y-Q-N-Y-V-A-R-S-E-G-NH_2
PAK 34-40	BB-Nle-A-S-A-L-A-S-V-NH_2
PAK 41-49	Ac-N-P-L-T-T-V-E-E-NH_2
	BB-Nle-N-P-L-T-T-V-E-E-NH_2
PAK 58-70	Ac-K-S-G-T-G-T-E-D-A-T-K-K-E-NH_2
	BB-Nle-K-S-G-T-G-T-E-D-A-T-K-K-E-NH_2
PAK 74-83	Ac-G-V-A-A-D-A-N-K-L-G-NH_2
	BB-Nle-G-V-A-A-D-A-N-K-L-G-NH_2
PAK 89-99	Ac-K-P-D-P-A-D-G-T-A-D-NH_2
	BB-Nle-K-P-D-P-A-D-G-T-A-D-NH_2
PAK 105-114	Ac-G-G-A-G-P-K-N-K-G-K-NH_2
	BB-Nle-G-G-A-G-P-K-N-K-G-K-NH_2
PAK 117-125	Ac-T-L-T-R-T-A-A-D-G-NH_2
	BB-Nle-T-L-T-R-T-A-A-D-G-NH_2
PAK 128-144	Ac-K-C-T-S-D-Q-D-E-Q-F-I-P-K-G-C-S-K-OH
	BB-Nle-K-C-T-S-D-Q-D-E-Q-F-I-P-K-G-C-S-K-OH

PAK A-129 BB-Nle-K-A-T-S-D-Q-D-E-Q-F-I-P-K-G-C-S-K-OH

PAK 3A Ac-A-C-A-A-D-Q-D-E-Q-F-I-P-K-G-C-S-K-OH

PAK 5A Ac-A-C-A-A-A-A-D-E-Q-F-I-P-K-G-C-S-K-OH

PAK 7A Ac-A-C-A-A-A-A-A-A-Q-F-I-P-K-G-C-S-K-OH

PAK 137-144 Ac-F-I-P-K-G-C-S-K-OH
BB-Nle-F-I-P-K-G-C-S-K-OH

PAK 121-144 Ac-T-A-A-D-G-L-W-K-C-T-S-D-Q-D-E-Q-F-I-P-K-G-C-S-K-OH
BB-Nle-T-A-A-D-G-L-W-K-C-T-S-D-Q-D-E-Q-F-I-P-K-G-C-S-K-OH

PAK 116-144 Ac-I-T-L-T-R-T-A-A-D-G-L-W-K-C-T-S-D-Q-D-E-Q-F-I-P-K-G-C-S-K-OH
BB-Nle-I-T-L-T-R-T-A-A-D-G-L-W-K-C-T-S-D-Q-D-E-Q-F-I-P-K-G-C-S-K-OH

PAK 100-110 NH_2-L-T-F-T-M-G-G-A-G-P-K-NH_2

PAK 92-110 NH_2-A-D-G-T-A-D-I-T-L-T-F-T-M-G-G-A-G-P-K-NH_2

PAK 82-110 NH_2-L-G-T-I-A-L-K-P-D-P-A-D-G-T-A-D-I-T-L-T-F-T-M-G-G-A-G-P-K-NH_2

PAO 58-70 Ac-S-K-I-K-I-G-T-T-A-S-T-A-T-NH_2
BB-Nle-S-K-I-K-I-G-T-T-A-S-T-A-T-NH_2

PAO 75-84 Ac-G-V-E-P-D-A-N-K-L-G-NH_2
BB-Nle-G-V-E-P-D-A-N-K-L-G-NH_2

PAO 88-99 Ac-V-A-I-E-D-S-G-A-G-D-NH_2
BB-Nle-V-A-I-E-D-S-G-A-G-D-NH_2

PAO 105-114	Ac-G-T-S-S-P-K-N-A-T-K-NH_2
	BB-Nle-G-T-S-S-P-K-N-A-T-K-NH_2
PAO 117-125	Ac-T-L-N-R-T-A-D-G-V-NH_2
	BB-Nle-T-L-N-R-T-A-D-G-V-NH_2
PAO 128-144	Ac-A-C-K-S-T-Q-D-P-M-F-T-P-K-G-C-D-N-OH
	BB-Nle-A-C-K-S-T-Q-D-P-M-F-T-P-K-G-C-D-N-OH

Ac=Acetyl group

Nle=Norleucine residue

BB=Benzoylbenzoyl

C. High-performance liquid chromatography (HPLC)

1. *Analysis of peptides by HPLC*

High-performance liquid chromatography (HPLC) is at present the most common technique used to purify and characterize peptides and proteins. The widespread application of HPLC is due to its sensitivity, speed and cost (Hermodson and Mahoney, 1983; Lazure *et al.*, 1990). The three main modes of HPLC used for peptide separations utilize differences in peptide size (size-exclusion HPLC or SEC), net charge (ion-exchange HPLC or IEC) or hydrophobicity (reversed-phase HPLC or RPC). RPC is the most widely used mode of HPLC because of its ability to separate closely related structures. In addition, RPC parameters such as pH, mobile phase or counterion can be varied to obtain optimum resolution of the peptide of interest from a complex mixture (Mant and Hodges, 1990). Thus, the quality of the syntheses and purity of the synthetic peptides were analyzed by RPC. A small sample of the crude peptide (1 mg or less) was brought up in a small volume (1 ml) of 0.05% aqueous TFA. The solution was centrifuged for 2-3 min in a bench top microfuge to sediment undissolved material prior to injection of the sample for HPLC analysis. A 10-20 µl sample was used for each analytical run.

The HPLC equipment consisted of a Spectra-Physics (San Jose, CA, USA) SP 8700 solvent delivery system and a SP8750 organizer module combined with a Hewlett-Packard HP1040A detection system, HP3390A integrator, HP85 computer, HP9121 disc drive and a HP 7470 A plotter. Samples were injected with a 0.5-2.0 ml injector loop (Model 7125, Rheodyne, Cotati, CA, USA). Alternatively, a Varian 5000 Vista series HPLC was used, coupled with a Waters Model M490 Programmable multiwavelength detection system. The TFA was redistilled prior to use. HPLC-grade acetonitrile was obtained from J.T. Baker (Phillipsburg, NJ, USA). Deionized water was purified by reverse osmosis using a HP661A water purifier (Hewlett-Packard, Avondale, PA, USA), or a Milli-Q Plus water system (Millipore, Bedford, MA, USA). HPLC-grade water available from J.T. Baker was used occasionally.

The chromatographic mode used for the purposes of this project was RPC. Elution of peptides during an analytical run was effected by an AB gradient (1% B/min) at a flow-rate of 1.0-2.0 ml/min where solvent A was 0.05% aqueous TFA and solvent B was 0.05% TFA in acetonitrile. The addition of TFA lowers the solvent to a pH of 2.0 suppressing the ionization of the free silanols on the silica support and provides the solvent with anionic counterions (trifluoroacetate) to ion-pair with positively charged basic residues of a peptide. The hydrophobicity of the TFA counterions can also further increase the affinity of the peptides for the reversed-phase sorbent. Apart from its effectiveness as an ion-pairing reagent, it also has the advantages of being volatile and UV transparent at low concentrations to permit monitoring of the peptide bond at 210nm (Guo *et al.*, 1987; Mant and Hodges, 1990). Another commonly used ion-pairing reagent is heptaflurobutyric acid (HFBA). HFBA is more hydrophobic than TFA, with which it shares many properties, but tends to exhibit baseline problems at 210nm. Acetonitrile is the favored organic solvent for RPC because of its UV transparency (to permit monitoring at 210nm), volatility and good resolution (Guo *et al.*, 1986; Mant and Hodges, 1986). A more non-polar solvent (e.g. isopropanol) or a more polar solvent (e.g., methanol) may be required for separations of very hydrophobic or very hydrophilic peptides, respectively (Mant and Hodges, 1990). Various gradient rates were used in preparative chromatography (0.2 - 1.0 % B/min), depending on the amount of crude peptide requiring purification and the perceived difficulty of the preparative separation.

The analytical reversed-phase columns used were: Aquapore RP-300 C-8, 220 x 4.6 mm internal diameter.(I.D.), 7-µm particle size and 300-Å pore size (Applied Biosystems Inc., Foster City, CA, USA); SynChropak RP-P C-18, 250 x 4.6 mm I.D., 6.5 µm, 300 Å, (SynChrom, Lafayette, IN, USA); Whatman Partisil C-8, 250 x 4.6 mm I.D., 5 µm, 60 Å . Silica-based packings of particle size 5 - 7 µm, containing octyl (C-8) or octadecyl (C-18) functionalities, provide powerful resolution of peptides. Small particle size packings require relatively low flow rates (1.0 ml/min) for satisfactory peptide

resolution. Although the length of these columns may vary, column length has little effect on peptide separation due to the mainly adsorptive-desorptive mechanism of peptide interaction with a reversed-phase sorbent (Guo et al., 1986; Mant et al., 1987). However, the column length has a direct effect on the loading capacity.

2. *Purification of Peptides by HPLC*

Following an analytical run of the crude peptide, the peptides were purified by HPLC. The crude peptide (30-50 mg) was dissolved in the smallest volume of starting buffer possible (approximately 5 ml). The highly concentrated peptide solution was centrifuged to sediment undissolved material. An analytical sample, 5-10 µl, was chromatographed to determine the total amount of peptide present. When the crude peptide contained hydrophilic and hydrophobic impurities with retention times close to the peptide of interest during the analytical run (1% B/min gradient rate), a shallow gradient of 0.2% B/min was employed in the preparative run (Mant *et al.*, 1987). The whole stock solution of 30-50 mgs was injected onto the column. Fractions (1ml) were collected and analyzed. Every third or fifth fraction was analyzed to identify the region on the chromatogram containing the peak of interest. Further analysis of the fractions within this region would then be carried out. The chromatogram of each run could be compared with the initial analytical run prior to purification to ascertain the peak of interest. In this way, the shoulders and neighboring peaks were eliminated, while fractions of interest were pooled and freeze dried. Dried peptides were stored in glass vials in a dessicator.

For larger samples of crude peptides, a semi-preparative column was used: SynChropak RP-P C-18 250 x 10 mm I.D., 6.5 µm particle size, 300Å pore size. Inexpensive 15-20 µm particle size packings may be used for purification when employing the shallow gradient elution procedure (Parker *et al.*, 1987). Semi-preparative columns are designed to be compatible with most analytical HPLC systems and are suitable for purification of small amounts of synthetic peptides.

D. Amino acid analysis

Samples of the freeze-dried peptides were analyzed for their amino acid composition by hydrolysis of the peptides in 500 µl of 6 N HCl *in vacuo* (Hirs *et al.*, 1954). The hydrolysis was carried out overnight in an oven at 110°C. The hydrolysates were dried and dissolved in citrate buffer (pH 2.2) and analyzed on a Durrum D-500 amino acid analyzer (Dionex, Palo Alto, CA, USA). With this analyzer, the separation of the amino acids is carried out on a cation-exchange column, first described by Spackman *et al.* (1958), using three different buffers. The acidic amino acids were eluted with 0.2 M sodium citrate buffer, pH 3.28; the neutral amino acids were eluted with 0.2 M sodium citrate, pH 4.25; the basic residues were eluted with 0.2 M sodium citrate buffer, pH 7.8, containing 1.1 M sodium chloride. Peptides containing cysteine residues were hydrolyzed at 160°C for 1 h with 6 N HCl containing 2% dimethylsulfoxide (Spencer and Wold, 1969). The cysteine content was then measured as the amount of cysteic acid in the hydrolysate. The ninhydrin method is used in the detection of the amino acids in the 10- to 100-nmole range. Ninhydrin reacts with amino acids in a transamination-decarboxylation reaction to yield Ruhemann purple (Bodanszky, 1988) which is monitored at 560nm. Proline, a secondary amine, reacts to give a yellow coloured compound which is monitored at 440nm. More sensitive (pmole range), but expensive, methods of detection such as fluorescamine or o-phthaldehyde have also been developed (Hughes and Frutiger, 1990).

E. Formation of an intrachain disulfide bond

In a number of peptides, it was necessary to oxidize the free sulfhydryl groups of the cysteine residues to form an intrachain disulfide bond. This was achieved in one of two ways. Firstly, the disulfide bond could be formed by air oxidation (White, 1961). In this method, the peptide was dissolved to a concentration of 0.1 mg/ml in a 100 mM ammonium bicarbonate solution (BDH, Poole, England), pH 8.3. The oxidation was carried out in an open flask by stirring the solution for 20-24 hours at room temperature.

The extent of oxidation could be verified by RPC or circular dichroism (see below). After completion of oxidation, the peptide solution was freeze-dried. The freeze-drying was repeated 2-3 times to remove all ammonium bicarbonate.

The second method involved a chemical reagent, potassium ferricyanide (ACS reagent grade, Aldrich, Milwaukee, WI, USA) to oxidize the free sulfhydryls and, hence, form the disulfide bond (Hope *et al.*, 1962). The peptides (5 mg) were dissolved to a concentration of 0.2 mg/ml in an aqueous solution of 2% acetic acid and the pH was adjusted to 6.8 with 10% aqueous ammonium hydroxide. Potassium ferricyanide (16 mg) was added to the solution which was then stirred for 30 min at room temperature. An additional 10 mg of potassium ferricyanide was added and the solution was stirred for another 30 min. Then 7 ml of wet ion-exchange resins (BioRad AG 1x4) were added to the solution and stirred for an hour. The resins adsorbed any unreacted potassium ferricyanide and the supernatant solution appeared as a clear solution. The resins were removed by passing the solution of peptide through pasteur pipets plugged with glass wool. The filtrate was then freeze-dried. The salts were removed by passing a solution of the freeze-dried sample through a reversed-phase column. In the absence of an acetonitrile gradient, the salts pass through the column, leaving behind the peptides adsorbed to the alkyl chains on the solid support. The peptides were then eluted by passing 30% solvent B (0.05% TFA in acetonitrile) through the column. The peptide fractions were collected and freeze-dried.

F. Modification of free sulfhydryls with NEM

The free sulfhydryl groups of cysteine residues were modified with N-ethylmaleimide (NEM), the use of which in detection of thiols has been previously described (Lunte and Kissinger, 1985). A sample of the peptide (100 µl) removed from an oxidation mixture containing 0.1 mg peptide per ml of ammonium bicarbonate was used for these assays, which have been previously described by Lee *et al.* (1990). The peptide was

reacted with 10 µl of NEM solution (1.0 mg/ml of 0.05% aqueous TFA) for 15 min at room temperature prior to HPLC analysis on an analytical reversed-phase column using a linear AB gradient of 1% B/min and at a flow-rate of 1ml/min, as described in Section II C on Analysis of peptides by HPLC.

G. Circular dichroism measurements

The polypeptide chain has a spatial assymmetry due to the constituent amino acids. In circular dichroism (CD), the ability of the peptides to act as optically active chromophores and display differential absorption of right and left circularly polarized light is dependent on the wavelength at which measurements are taken (Freifelder, 1982). Circular dichroism measurements have been used to verify the structure of cyclic peptides (Matsoukas *et al.*, 1984).

In these studies, reduced and oxidized forms of AcPAK 128-144 were dissolved separately to a concentration of 1mg/ml in 50 mM potassium phosphate buffer, pH 7.0. The peptide solution was put into a CD cell of 0.503 cm pathlength and scanned at a rate of 5 nm/cm. The wavelength range for the scan was 190 to 350 nm. The CD spectra were recorded on a JASCO J-5000 Spectropolarimeter attached to a JASCO DP-500N data processor and a Lauda (Model RMS) water bath to control the temperature of the cell. The oxidized AcPAK 128-144 was reduced with 1 mM dithiothreitol and the process was repeated.

H. Preparation of peptide conjugates

1. *Peptide-carrier conjugates*

The preparation of peptide-carrier conjugates was achieved by a photochemical reductive method (Parker and Hodges, 1985). The photoreactive moiety used was benzoylbenzoic acid (BBA) (Aldrich, Milwaukee, WI, USA) which was attached to the N-terminal of the peptides using the standard SPPS coupling procedure (Merrifield, 1963)

with the symmetric anhydride of the benzoylbenzoyl acid (Parker and Hodges, 1985). The coupling procedure has been described under Section II B.5. The photoreactive benzophenones were first introduced by Galardy *et al.* (1973, 1974) for photoaffinity labelling experiments. The reagent is stable and inert in the presence of water. This is important as it allows purification of the benzoylbenzoyl peptides. Another advantage of using BBA in coupling peptides to protein carriers is that the peptides are anchored to the carriers specifically via the N-terminal end. The use of chemical bifunctional crosslinkers does not permit such a defined coupling of peptides to carriers. A scheme of the photochemical covalent linkage of the benzophenone to a ligand is shown in Figure II.1. The photoactivated species will react with primary, secondary and tertiary carbons along with various sidechains of a protein (Galardy *et al.*, 1974; Parker and Hodges, 1985). Two protein carriers were used: keyhole limpet hemocyanin (KLH) and bovine serum albumin (BSA, RIA grade), both purchased from Sigma Chemical Co., St. Louis, MI, USA. The KLH-peptide conjugates were used for immunization as it is a good immunogen (Harlow and Lane, 1988). Stock solutions of 1g BSA per ml in 50 mM phosphate buffer and 100 mg KLH per ml in 0.1 M ammonium bicarbonate were prepared. The KLH solution was made up by addition of aliquots of the buffer and allowing it to be soaked up by the protein. Otherwise clumping would occur.

The preparation of the peptide conjugates has been described (Lee *et al.*, 1989). Peptides (3-5 mg) to be conjugated were added into a 10 x 75 mm glass tube and dissolved in 10 µl of water or phosphate buffer. A bigger volume may be necessary to dissolve more hydrophobic peptides. Then 10 µl of BSA stock solution or 100 µl of KLH stock solution was added into the glass tube. The mixture of peptide and carrier protein were irradiated at 350 nm with a RPR 208 preparative reactor (Rayonet, Southern New England Ultraviolet Co., Middletown, CT, USA) for an hour in the cold room at 4 °C. Any free unconjugated peptide removed by dialysis. The photolyzed conjugate mixture was brought up in 3-5 ml

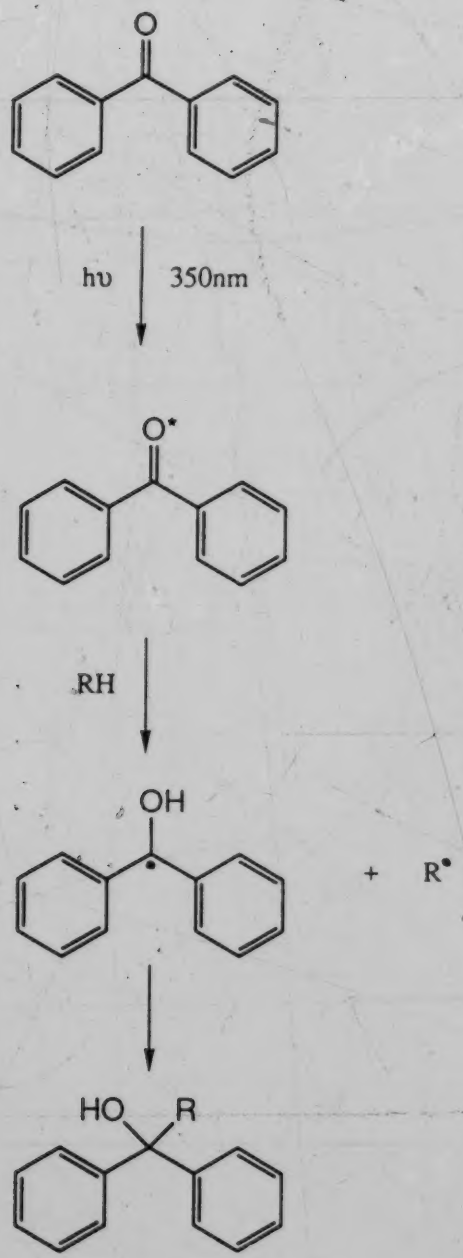


Figure II 1. A scheme of the conjugation mechanism of photoactivated benzophenone to a primary carbon (R•)is shown.

of 8 M urea and dialyzed against water containing 6 M urea in a cold room at 4 °C. The dialysis tubing has a molecular weight cutoff of 12,000 to 14,000 (Spectra/Por, Fisher Scientific, Edmonton, Canada). Subsequent dialyses in 1 M urea, 100 mM ammonium bicarbonate and 10 mM ammonium bicarbonate were carried out before the peptide conjugate solution was removed and freeze-dried.

The molar ratio of peptide coupled to the protein carrier was determined by quantitation of norleucine by amino acid analysis of the peptide-conjugates. The norleucine was the penultimate residue on the peptide chain (the BBA moiety is located at the N-terminal). It acted as a spacer between the peptide and carrier protein and also provided for a way of determining the ratio of the peptide carrier which was hydrolyzed (see Section D above) and the nanomolar quantity of norleucine determined. The ratio was then determined by comparing the quantity of a particular amino acid on the carrier which is not found on the sequence of the synthetic peptide coupled to the carrier. Two or three such analyses were carried out to obtain a ratio that reflected the average number of norleucine residues per carrier. Since the numbers of residues of the selected amino acids are known (from published data and verified by an in-house analysis of the amino acid composition of the protein carrier molecule), the ratio of peptide to carrier was represented by the averaged ratio of the norleucine with the selected amino acid. The procedure detailed here generally resulted in ratios of 3-5 moles of peptide per mole of carrier.

2. *Peptide-enzyme conjugates*

The methodology for preparing peptide-enzyme conjugates was the same as described above. Alkaline phosphatase (bovine intestinal mucosa, Type VII-NL, Sigma) was dissolved in a minimal volume of PBS together with the peptides and irradiated for 30 min instead of 60 min. The alkaline phosphatase-peptide conjugate was brought up in 0.5 to 1.0 ml of PBS and dialyzed against PBS in the cold room at 4 °C to remove any free peptides. The dialysate was aliquoted into centrifuge tubes and frozen until ready to be used.

I. Rabbit immunization

A comprehensive practical manual on the production of antibodies is found in a recent publication by Harlow and Lane (1988). Eight week old female rabbits (crossed between Flemish and giant lobe ear Dutch rabbits) were purchased from the animal research facility at the University of Alberta, Edmonton, Canada. The rabbits were immunized with 500 µg of KLH-peptide conjugates dissolved in 700 µl of a 1:1 mixture of Freund complete adjuvant (Gibco Laboratories, Grand Island, NY, USA) and 50 mM phosphate buffer saline. The injections were given subscapularly (2 x 200 µl) and intramuscularly (1 x 300 µl) and two rabbits were used for each conjugate. The rabbits were boosted after two weeks with the same amount of peptide conjugate in incomplete Freunds adjuvant. Rabbits were bled two weeks later. A small volume of rabbit blood was removed with a 21" gauge needle from the biggest vein on the ear of the rabbit. The advantage of the crossed rabbits was the ease with which blood could be removed because of the large veins on the ears.

J. Enzyme-linked immunosorbent assay

Antibody titers from rabbit sera were determined by direct enzyme-linked immunosorbent assays (ELISA). This method has been pioneered by Engvall and Perlmann (1971, 1972) and its principles reviewed by Voller *et al.* (1976. The presence of antipeptide antibodies was screened using the BSA-peptide conjugates and not the KLH conjugates, which were used as the immunogens. We were interested in the antibodies directed against the peptides and not the carrier molecule. A stock solution of the BSA-peptide conjugate consisted of 1 mg/ml of coating buffer (0.015 M sodium carbonate buffer, pH 9.8, with 0.02% sodium azide). The stock solution was diluted to the appropriate concentration with the coating buffer. In these assays, a 5 µg/ml concentration of the antigen was used and 100 µl aliquots of this solution were added into the wells of the microplate. The 96-welled plates were purchased from Terochem, Edmonton, Canada.

The plates were kept overnight in a moist chamber (plastic containers with soaked paper towels) at 4°C to coat effectively the surfaces of the wells with these antigens. The wells were washed three times with phosphate buffer saline, pH 7.4 (1.5 mM KH_2PO_4 3.2 mM Na_2HPO_4, 2.7 mM KCl, 0.136 M NaCl) containing 0.05% Tween 20 (PBST). The wells were dried by shaking off the wash buffer. Dilutions of the antipeptide antisera were prepared using a 3-fold serial dilution with PBST-BSA. The antibody diluting buffer, PBST-BSA, was prepared by dissolving 2g BSA (RIA grade, Sigma Chemical Co., St. Louis, MO, USA) in 100 ml PBST. A 100 µl aliquot of the diluted antiserum was added to each well and incubated in a moist chamber for 2 h at room temperature. Excess unbound antibodies were removed with three washes of PBST. The goat anti-rabbit IgG (antibody-alkaline phosphatase [calf intestine] conjugate, from Boehringer Mannheim Biochemicals, Indianapolis, IN, USA) was diluted (1:1000) with PBST and 100 µl aliquots were added to the wells. The plates were replaced into the moist chamber and incubated at room temperature for another 2.5 h. The wells were washed three times with PBST. The enzyme substrate tablets, *p*-nitrophenylphosphate (Sigma Chemical Co., St. Louis, MI, USA), were dissolved in 10% diethanolamine buffer, pH 9.8, to give a 1 mg/ml concentration. Aliquots of 100 µl were added to the wells and a yellow coloration resulting from the enzymatic breakdown of the substrate was monitored at 405 nm with a Titertek Multiscan microplate reader (Flow laboratories Inc., Mississauga, Ontario, Canada). Readings were taken at 15 min, 30 min and 60 min.

In indirect or competitive ELISA, the wells of the plate were coated with the antigen of interest, and a few modifications of the above procedures were made. Instead of adding diluted antipeptide antibodies into the wells, a known concentration of the IgG or Fab was preincubated overnight at 4°C with serially diluted competing antigens in centrifuge tubes. Aliquots of the antibody-antigen mixture were then added to the precoated wells on the microplate and incubated for 2 h at room temperature. Subsequent steps were the same as those used in direct ELISA. The selection of concentration range of antibodies and

competing antigen were obtained by carrying out a checker board type experiment. In this approach, a few selected concentrations of the antibody were used and each concentration of the antibody was incubated with different concentrations of the competing antigen. A control with no competing antigen present was used as a reference. The concentration of antibody that gave a marked inhibition in binding (in the range of competing antigen concentration) was selected. A more extensive concentration range of antigen was further used in the competitive assays to give rise to maximum inhibition of binding. The percent inhibition was determined by the following equation:

$$\frac{(OD_O - OD_C)}{OD_O} \times 100 = \% \text{ Inhibition}$$

where OD_O is the absorbance without competing antigen

and OD_C is the absorbance in the presence of competing antigen

K. Purification of immunoglobulin G (IgG)

Immunoglobulin G was purified using a Protein A-Sepharose CL-4B column (Pharmacia). The antiserum (1.5 ml) was filtered by passage through a 0.45 μm filter (Millipore) attached to the end of a syringe. The antiserum was loaded onto the protein-A column which was pre-equilibrated with 0.1 M phosphate buffer (PB). The column was washed with three column volumes of PB. Protein A-bound immunoglobulins were eluted with 1.0 M acetic acid or 0.1 M glycine-HCl buffer, pH 3.0. Fractions of 1 ml were collected and the protein content monitored spectrophotometrically at 280 nm. Each fraction (diluted 1:100) was assayed using dorect ELISA. Fractions with high OD readings at 405 nm were pooled and dialyzed against distilled water in the coldroom. The dialysate with IgG was freeze-dried and stored at -20°C until ready for use. The protein-A column

was washed with three column volumes of phosphate buffer containing 0.1% sodium azide and stored at -4°C.

L. Preparation of Fab fragments from IgG

The preparation of Fab fragments from IgG using papain has been reported by Porter (1959). The digestion buffer (pH 7.2) consisted of 100 mM Na_2HPO_4, 10 mM cysteine-HCl and 2 mM EDTA. The IgG was dissolved in the digestion buffer together with papain (papaya latex type III, Sigma) to a ratio of 1:100 (w/w). The digestion mixture was stirred overnight at 37°C. The reaction was terminated by the addition of iodoacetamide to give a final concentration of 0.15 M. The digestion mixture was freeze-dried and dissolved in a small volume (~1 ml) of elution buffer. This digest was loaded into a Sephadex G-100 column (1.0 x 22.5 cm) pre-equilibrated with a Tris-phosphate buffer containing 6.7 mM Na_2HPO_4, 10 mM Tris-HCl, 175 mM NaCl, pH 7.4. Fractions (12 drops per tube) were collected on a LKB 7000 Ultrarac fraction collector. The fractions were analyzed for protein content (OD_{280}) and Fab content. Two protein peaks were noted and the first peak contained the antigen-binding Fab fragments, detected by direct ELISA. The fractions with Fab present were pooled and further purified on the Protein A-sepharose column. Unbound Fab fragments were eluted with the PB washes while undigested IgG was retained on the column. The Fab fractions were dialyzed against distilled water and freeze-dried.

M. Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was carried out according to the Laemmli method (Laemmli, 1970) using 15% polyacrylamide running gels. The BioRad Mini Protean II gel apparatus (BioRad Laboratories, Richmond, CA, USA) was used for the gel electrophoresis. Glass plates were cleaned and clamped together with the appropriate spacers. The running gel was prepared by mixing 7.6 ml of a 44% acrylamide solution (containing 0.8%

bisacrylamide) with 0.55 ml of ammonium persulfate (AMPS) (10 mg/ml), 0.45 ml of 10% SDS (w/v), 8.45 ml 1 M Tris (pH 8.8) and 5.4 ml 0.4% NaCl. Crosslinking of the acrylamide was catalyzed by the addition of 20 µl of N,N,N',N'-tetra methylethylenediamine (TEMED) (BioRad Laboratories, Richmond, CA, USA). Because the polymerization begins immediately after the addition of TEMED, the preparation was quickly poured between the plates. A space of 1/4-1/2" from the top of the glass plates was left for the stacking gel. The mixture was covered with propanol to prevent drying while the polymerization was occurring. The above mixture should give a 15% crosslinking in the gel. The percentage crosslinking could be varied by changing the volume of acrylamide added and compensating for this by a corresponding change in the volume of sodium chloride.

After the running gel has polymerized and the propanol been drained, the stacking gel was poured on top of it. The 7% crosslinking stacking gel was prepared by mixing 2.25 ml of a 30% acrylamide solution (containing 0.8% bisacrylamide), 0.21 ml of AMPS (10 mg/ml), 0.09 ml 10% SDS (w/v), 1.25 ml TM Tris (pH 8.8), 5.7 ml 0.4% NaCl and 20 µl TEMED. A comb was inserted into the stacking gel during polymerization.

Samples to be analyzed (2-5 µg) were boiled with 20 µl of 'Blue Juice' for 3 min. The Blue Juice contained 1.25 ml of 0.25 M Tris (pH 6.8), 1 ml 10% SDS, 1 ml 50% glycerol, 200 µl 2-mercaptoethanol, 200 µl 0.5% Bromophenol blue and 350 µl water. Samples were then loaded with a Hamilton syringe onto the slots in the stacking gel. The gel was then placed onto the electrophoresis apparatus. The electrode buffer consisted of 0.25 M Tris, 2 M glycine and 10% SDS (w/v). The buffer was diluted 10-fold prior to the electrophoretic runs. The gel was subjected to electrophoresis for 4 h at 40 mA constant current supply using an Electrophoresis constant power supply (ECDS 2000/300 from Pharmacia).

The protein bands on the gel could be stained for visualization or electrophoretically transferred onto nitrocellulose paper for immunoblot assays. Coomassie Blue stain

solution consisted of 5 g/litre Coomassie Brilliant Blue R (Sigma), 25% isopropanol and 10% acetic acid. The gel was placed in the stain for at least 2 h. The gel was destained in a solution containing 10% acetic acid and 10% methanol.

N. Immunoblot assays

In these assays, proteins separated by SDS-PAGE are electrophoretically transferred onto nitrocellulose membranes. The procedure used has been pioneered by Towbin *et al.* (1979). The slab gel was removed and laid together with a wet sheet of nitrocellulose membrane (BioRad). Two sheets of wet blotting filter paper were used to sandwich the gel and nitrocellulose membrane. All trapped air bubbles were removed. This was then placed between two pieces of scouring pad and secured by plastic grid gel holders. The pad and gel holder come together with the Trans-Blot Electrophoretic Transfer Cell (BioRad Laboratories, Richmond, CA, USA). The gel was placed into the chamber of the transfer cell with the nitrocellulose sheet facing the anode and the gel facing the cathode. The transfer buffer consisted of 25 mM Tris, pH 8.3, 192 mM glycine and 20% (v/v) methanol. Electrophoretic transfer, which was carried out in the coldroom, took 4 h under a 0.2 Amp current supplied by a BioRad constant voltage power supply (Model 250/2.5).

The transfer of proteins from gel to nitrocellulose could be visualized as the molecular weight standards on one of the lanes in the gel had been prestained. The prestained molecular weight markers were supplied by Bethesda Research Laboratories (Gaithersburg, MD, USA). The nitrocellulose sheet was transferred to a 3% solution of gelatin-tris buffered saline (TBS) for an hour to block all excess protein binding sites on the nitrocellulose sheet. The TBS solution consisted of 20 mM Tris, 500 mM NaCl, pH 7.5. After the blocking step, the sheet was washed twice (2 and 15 min) with 0.05% Tween-20 in TBS (TTBS). The nitrocellulose sheet was placed into a plastic bag and sealed, or placed onto a small tray containing diluted antiserum and agitated overnight at room

temperature. The antiserum had been diluted with 1% solution of gelatin-TTBS. The nitrocellulose sheet was then washed with TTBS (2 x 10 min). A second antibody solution containing goat anti-rabbit IgG alkaline phosphatase conjugate, diluted 1:3000 with a 1% gelatin-TTBS, was incubated at room temperature for an hour with the nitrocellulose sheet. The nitrocellulose membrane was removed and washed with TTBS (2 x 10 min) and a final wash with TBS.

During the final wash steps, the colour development solutions were prepared. The *p*-nitro blue tetrazolium chloride (NBT) was dissolved to a concentration of 15 mg/ml in 70% aq DMF and the 5-bromo-4-chloro-3-indolylphosphate-toluidine salt (BCIP) to a concentration of 7.5 mg/ml in DMF. One ml of each of these enzyme sustrates was mixed into 50 ml 0.1 M sodium carbonate buffer, pH 9.8, containing 1 mM $MgCl_2$. This was prepared only when the nitrocellulose was ready to be developed. The enzymatic reaction with these chemicals resulted in the appearance of purple-red bands. These assays were carried out with an immunoblot assay kit available from BioRad Laboratories. The instructions for this assay come together with the purchase of the assay kit.

O. Buccal epithelial cells (BECs)

BECs were collected into 20 ml of chilled PBS with wooden applicator sticks from healthy, non-smoking, male volunteers (n=10). BECs were removed from the applicator sticks by agitation in phosphate buffered saline, pH 7.2 (PBS), as described by McEachran and Irvin (1985). The BECs were washed 3 times (2 000 x g for 10 min at 4°C) with 10 ml of PBS, then passed through a 70 µm nylon mesh. The cell concentration for the BECs was determined with a hemocytometer and the BEC concentration adjusted to 2.0×10^5 cells/ml.

P. Inhibition of pilus binding to BECs with Fabs

An equal volume of Fab fragments specific for the various synthetic peptide-BSA conjugates in PBS (0.1 ml at 0.3-0.61 mg/ml), were added to purified PAK pili (0.1 ml at 100 µg/ml) and incubated at room temperature for 30 min (Lee *et al.*, 1989). To this mixture, BECs (0.2 ml at 2.0 x 10^5 BECs/ml) were added and the mixture was incubated at 37°C, with shaking at 300 rpm in a New Brunswick gyroshaker. After 2 h, BECs were collected by centrifugation (13 000 x g for 2 min) and washed 5 times with PBS to remove excess Fab and unbound PAK pili. The PAK pili bound to the BECs were visualized using a PAK pili-specific monoclonal antibody, PK3B, developed by Irvin and coworkers (Doig *et al.*, 1989). The procedure to visualize this binding is as follows. Monoclonal antibody PK3B (0.2 ml of a 10^{-4} dilution in PBS) was added to the BEC pellet and the incubation was repeated for one hour. The BECs were then collected by centrifugation and washed 5 times with PBS. Goat anti-mouse IgG Fc-specific immunoglobin G-peroxidase conjugate (Jackson Laboratories) was added to the BEC pellet (0.2 ml of a 1 in 5000 dilution) and the mixture incubated as described above for 30 min. The BECs were collected by centrifugation and washed 5 times with PBS. The pellet was suspended in 0.2 ml of ABTS (2,2'-azido-di-(3-ethylbenzthiazoline sulfonic acid) in citrate buffer, pH 4.2, containing 0.03%(v/v) hydrogen peroxide and transferred to a clean tube. The reaction was stopped by the addition of 0.2 ml of 4 mM NaN_3 and the optical density at 405 nm was determined after removal of the BECs by centrifugation. The BEC concentration in each tube was determined with a hemocytometer at the end of the assay prior to the removal of BECs by centrifugation.

Q. Inhibition of bacterial binding to BECs

The procedure for the following assay has been previously described (Doig *et al.*, 1988; McEachran and Irvin, 1985). Fab fragments from monoclonal IgG (100 ug/ml) were added to 0.1ml of ^{35}S-labelled bacteria (0.7 x 10^8 to 2.0 x10^8 colony forming

units/ml) in PBS and incubated for 30 min at room temperature. Then, PBS (0.4ml) and either 1.0 ml of BECs (2.0 x 10^5 cells/ml) or 1.0 ml of PBS (to assess nonspecific binding of bacteria to the filters) was added. The mixture was incubated at 37 °C with shaking at 300 rpm for 2h. BECs with bound bacteria were collected by filtration on 12-µm pore-size polycarbonate filters (Nucleopore Corp., Pleasanton, CA, USA) that had been pretreated with 2% (w/v) BSA in PBS to reduce nonspecific binding. The filters were washed with 15 ml of PBS and dried in scintillation vials. Aquasol (5 ml) was added to each vial and the radioactivity determined by scintillation counting in a Beckman LS-150 liquid scintillation counter. All assays were performed in triplicate. Epithelial cell concentration was determined at the end of the assay to correct for cells lost during the incubation and experimental steps.

R. AcPAK 128-144 peptide binding to BECs

Aliquots of 0.2 ml BECs (2.0 x 10^5 cells/ml) were added to an equal volume of synthetic peptide (0 to 120 nmol/ml) in PBS and incubated for an hour at 37°C with agitation at 300 rpm as previously described (Irvin *et al.*, 1989). The cells were washed five times with PBS by centrifugation (13,000 x g for 2 min) at room temperature to remove unbound peptides. The synthetic AcPAK 128-144 peptide bound to BECs can be visualized using a monoclonal antibody, PK99H. This MAb, developed by Doig *et al.* (1990), has been shown to bind the AcPAK128-144 peptide. The visualization of peptide binding is as follows. The monoclonal antibody, PK99H (0.2 ml of a 10^{-3} dilution) was added to the BEC pellet and the incubation repeated. The cells were collected by centrifugation and washed five times with PBS. Goat anti-mouse heavy and light chain IgG-peroxidase conjugate (Jackson Laboratories) was added to the BEC pellet (0.2 ml of a 10^{-4} dilution) with the subsequent steps in the assay carried out as described above (see Section II P).

S. AcPAK 128-144 peptide inhibition of pilus binding to BECs

The reduced peptide was used in these assays. BECs (0.2 ml at 2.0×10^5 cells/ml) and the AcPAK 128-144 peptide (0.1 ml, for a final concentration of 0, 40, 80, or 120 nmol/ml) were preincubated for 30 min at room temperature. Pili (0.1ml at 0 to 100 µg/ml) were added to the BECs with various concentrations of synthetic peptides. The mixtures were incubated for 2 h at 37°C during agitation at 300 rpm. BECs and bound pili were collected by centrifugation and washed five times with PBS to remove unbound pili as described above. Monoclonal antibody, PK3B (0.1ml of a 10^{-4} dilution), which is known to bind pili but not the synthetic peptide, was added to the BECs with bound pili and incubated for 1h. The remainder of the whole cell assay is described in Section II P (also see Irvin *et al.*, 1989).

T. Binding of alkaline phosphatase-peptide conjugate to PAK pili

The wells of the microplate were coated with 100 µl of the PAK pili (10 µg/ml) by incubation overnight in the coldroom. Excess pili were removed by aspiration and free protein binding sites on the wells were blocked with 200 µl of BSA (5 g/ml) for an hour at 37°C. Dilutions of the peptide-enzyme conjugates were made using PBS containing 0.05% BSA. The diluted conjugate solutions (100 µl) were added to the wells in quadruplicates and incubated at 37°C for an hour. The wells were washed five times with 200 µl PBS containing 0.05% BSA. Finally, 100 µl of *p*-nitrophenylphosphate (1 mg/ml) was added to the wells. The reaction was quenched after 30 min by the addition of 50 µl of 2N NaOH. Absorbance readings at 405 nm were recorded.

U. Binding of alkaline phosphatase-peptide conjugate to BECs

The BECs were collected as described above in Section II O. The incubation mixture consisted of 1.0 ml of BECs (2.0×10^5 cells/ml), 0.1 ml diluted peptide-conjugate (10^{-2}, 5×10^{-3}, 10^{-3}, 5×10^{-4}, 10^{-4} dilution) and 0.1 ml of a 5% BSA solution. The

incubation was carried out at 37°C with agitation of 300 rpm. Then the cells and bound conjugates were harvested by centrifugation (13,000 x g for 1 min). Unbound conjugates were removed by washing the pellets seven times with 0.5 ml PBS. The pellets were suspended in 0.5 ml PBS and transferred to fresh centrifuge tubes. The PBS was removed and 600 µl of substrate solution was added to the BECs and incubated for 30 min with gentle agitation. The reaction was quenched with 150 µl of 2N NaOH. The cells were sedimented by centrifugation and aliquots (200 µl) of the supernatant solution were added to wells on a microplate. Absorbance readings were then recorded at 405nm.

Chapter III. Development of a Method to Verify and Purify by Reversed-Phase Chromatography Intrachain Disulfide-Bridged Peptides for Preparation of Synthetic Peptide Vaccines

A. Introduction

This research project involved the synthesis and purification of a large number of peptides for preparation of peptide-carrier conjugates for use in immunization and other assays. One of the problems encountered earlier in this work was how to verify the formation of an intrachain disulfide-bridged (oxidized) peptide as opposed to the reduced conformer. The standard procedure for checking the completion of an oxidation process often involves the use of a spectrophotometric method involving a reagent, 5,5'-dithiobis-2-nitrobenzoic acid (DTNB), commonly referred to as Ellman's reagent (Ellman, 1959), to detect the presence of reduced peptide. This chapter deals with the development of a rapid and easy quantitative method to verify the presence of oxidized and reduced peptides by reversed-phase high-performance liquid chromatography (RPC). In addition, this chapter also describes the synthesis, purification and characterization of peptides, including the resolution by RPC of a large number of peptides in their reduced and oxidized forms. The free sulfhydryl or cysteine-containing peptides will be referred to as "reduced" and the intrachain disulfide-bridged form of the peptides as "oxidized". RPC was used to monitor the extent of oxidation of peptides over a time period. The resolution of reduced and oxidized peptides by RPC, following modification of the free thiol (-SH) groups of the reduced peptides with -SH specific reagents, is also examined.

With regards to the prevalent use of synthetic peptides in current biological problems, and the common occurrence of disulfide bonds in peptides and native fragments of proteins, it is important to be able to distinguish the reduced peptide from the intrachain disulfide bridged peptide prior to their use in biological assays. As we shall see later in this project, the presence of a disulfide bond in the *Pseudomonas* pilin protein that we are

studying was found to be important for its function. Synthetic peptides corresponding to the region of the protein molecule containing the disulfide loop mimicked the function of the protein. In terms of development of a *Pseudomonas* vaccine that will be effective against a whole spectrum of *P. aeruginosa* strains, it would seem that the disulfide bridge at the C-terminal of the pilin protein would be a requirement. Production of large amounts of the peptide would be necessary and the integrity of the oxidized peptide would have to be assessed and verified. It is felt that the approach using RPC could be one of the methods useful in the verification of the quality of oxidized peptide in order to achieve a maximum effect as a vaccine. Its application towards solving other biological problems would be to provide an easy way of verifying the absence or presence of the disulfide bond in the peptide, thereby enabling researchers to determine the importance of the intrachain disulfide bridge to the function of the peptide. The method that has been developed here is sufficiently fast and easy to be employed in such investigations. It does not require any more sophisticated equipment other than a HPLC instrument, together with a UV detection system, which is routinely used for the purification of peptides.

B. Results

1. *Peptide synthesis*

The synthesis of peptides using the well established method of choice, the Merrifield solid-phase procedure (Merrifield, 1963), generally gave rise to good syntheses. The quality of the synthetic peptides obtained from the Beckman 990 synthesizer varied between peptides. Even though the Beckman 990 peptide synthesizer is a 20-year-old instrument and the speed of synthesis is a lot slower than state-of-the-art equipment such as the Applied Biosystem model 430 peptide synthesizer, it still offered efficient syntheses and large product yields. Representative examples of both good and moderately good syntheses are shown in Figures III.1 and III.2, respectively. In a good synthesis (Figure III.1), one major peak is observed on the HPLC chromatogram, together with a few relatively minor components. Figure III.2A shows an example of a major peak of interest and a few major contaminant peaks. The peak of interest was purified by RPC using a shallow gradient (0.2% B/min) on an analytical RPC column, as described by Mant *et al.* (1987). A 70-mg sample of the AcPAK 121-144 peptide was dissolved in a minimal volume of 0.05% aqueous TFA and applied onto the column. An analytical chromatogram of the solution was first obtained (Figure III.2A). Fractions were collected during the preparative run (Figure III.2B). Fractions were analyzed by RPC and those containing the peptide of interest were pooled. Figure III.2D shows the chromatogram of pooled fractions of the peptide of interest. Figures III.2C and III.2E show the chromatograms of pooled fractions before and after the peak of interest. Only a small quantity of the peptide of interest was lost in this purification, and a yield of 8 mg of purified peptide was obtained. Although the peptides were not always 100% pure, they were of adequate purity for our purposes. The composition of the peptides was verified by amino acid analysis.

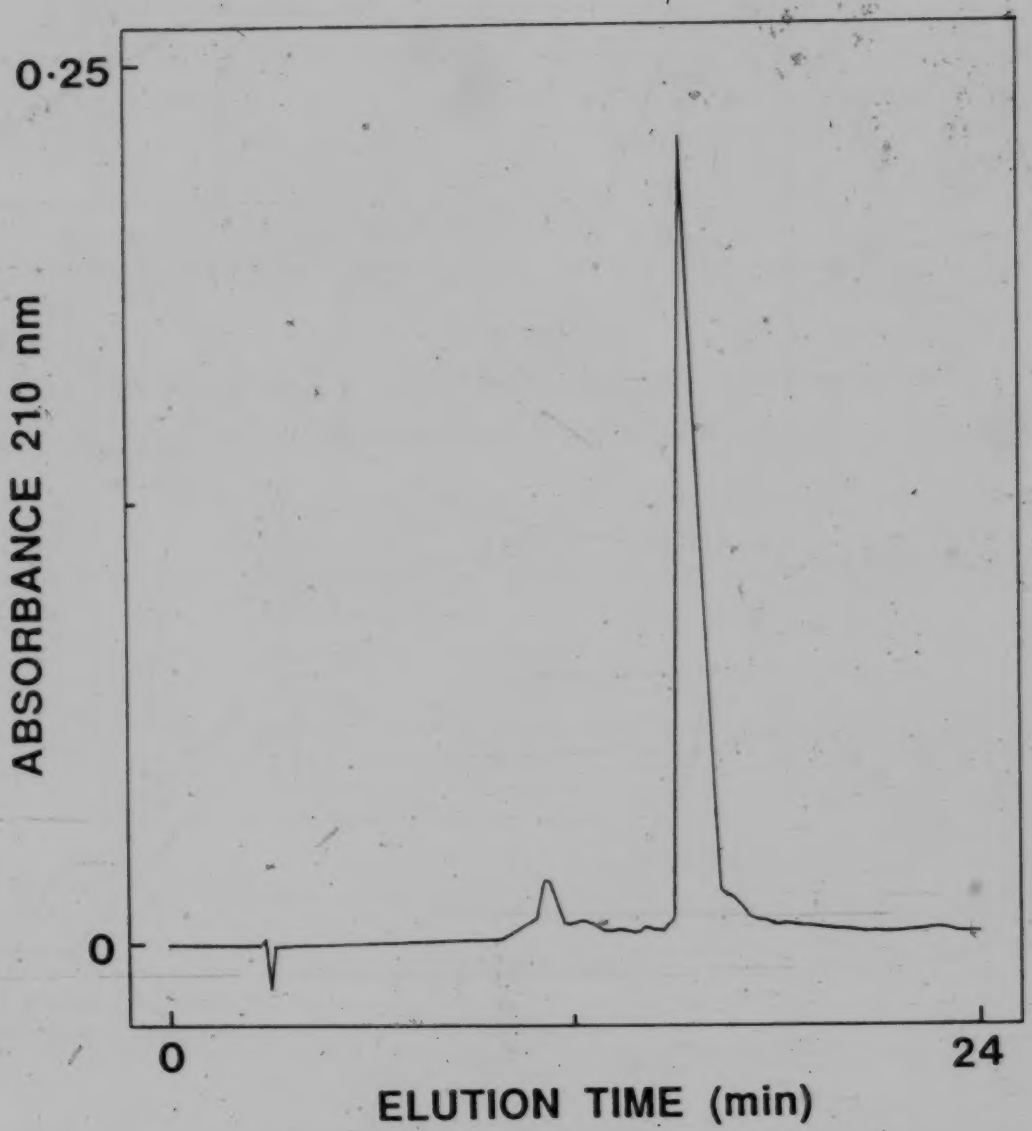
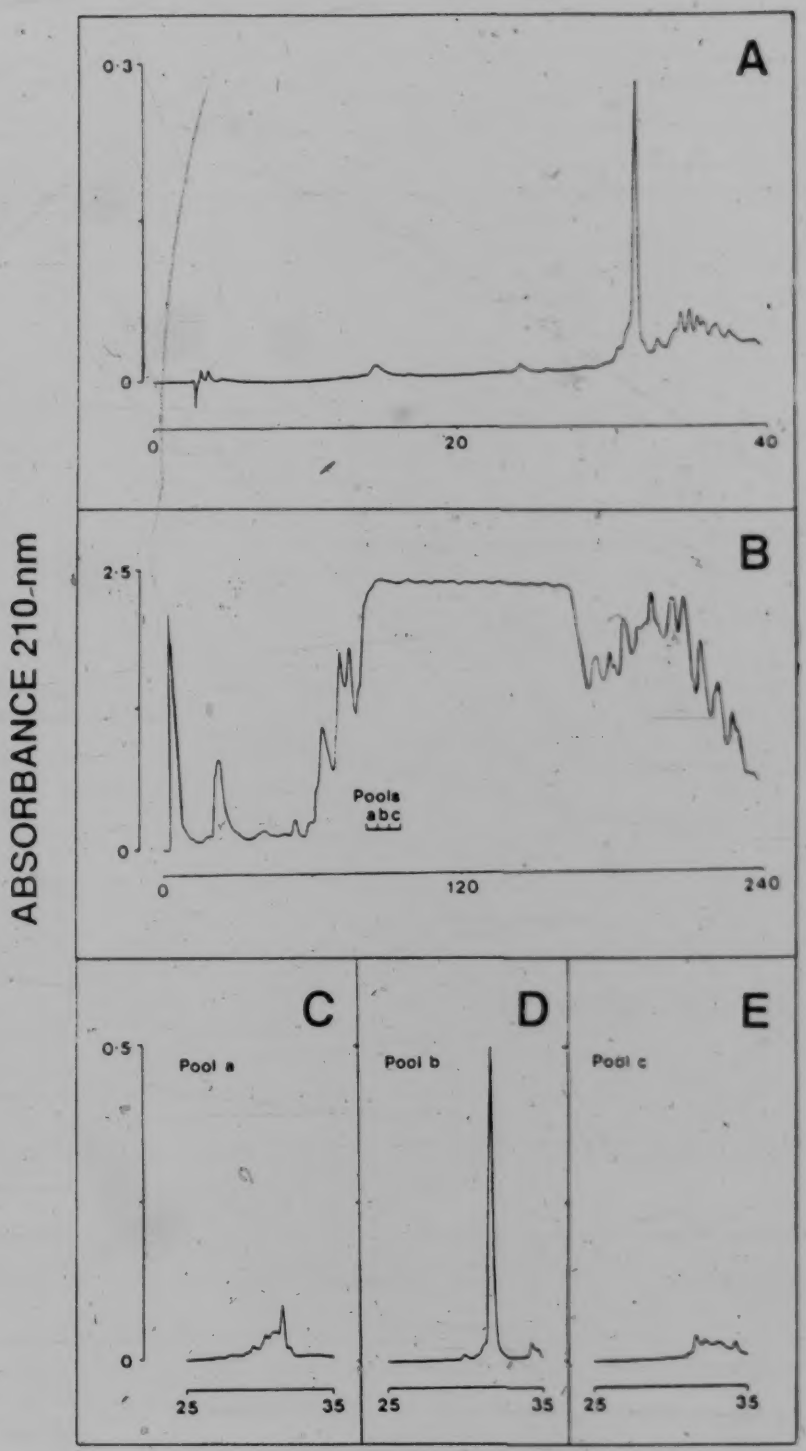


Figure III.1. HPLC profiles of synthetic AcPAK 58-70 peptide (crude) chromatographed on an analytical Whatman Partisil C-8 column (250 x 4.6 mm i.d.); Flow-rate: 1 ml/min; linear AB gradient of 1% B/min where eluent A is 0.05% aqueous trifluoroacetic acid (TFA) and eluent B is 0.05% TFA in acetonitrile.

Figure III.2. Purification of synthetic AcPAK 121-144 peptide on an analytical Aquapore RP-300 C-8 column (220 x 4.6 mm i.d.). A is the analytical run of the crude peptides to be purified; B is the chromatogram of the preparative run; C, D, and E are runs of pooled fractions from the preparative run. The conditions for A, C, D, and E are as follows: linear AB gradient increasing 1% B/min at 1.0 ml/min where eluent A is 0.05% aqueous TFA and eluent B is 0.05% TFA-acetonitrile. The conditions for the preparative run are as follows: linear AB gradient increasing 0.2% B/min at 1.0 ml/min where eluent A is 0.05% aqueous TFA and eluent B is 0.05% TFA-acetonitrile.


A
0·3
0
0
20
40
B
2·5
0
Pools
abc
0
120
240
C
0·5
0
Pool a
25
35
D
Pool b
25
35
E
Pool c
25
35
ABSORBANCE 210 nm
ELUTION TIME (min)

2. *The oxidation of peptides: formation of an intrachain disulfide bond*

Initially, two methods of oxidation (potassium ferricyanide and air-oxidation) were employed to form intrachain disulfide bonds. Both procedures were equally effective in the formation of the intrachain disulfide bond, as judged by the circular dichroism data (see below). Air-oxidation in ammonium bicarbonate is a very convenient approach, since the buffer is volatile. After the oxidation was completed, repeated freeze drying removed all the ammonium bicarbonate, leaving behind the oxidized product. The convenience of this method made it the method of choice. In the potassium ferricyanide method, a number of desalting or dialysis steps were required. However, time of oxidation of this procedure was shorter than the air-oxidation approach, since as the oxidizing agent was more reactive than oxygen.

The reduced and oxidized AcPAK 128-144 conformers were not resolved by RPC. This is illustrated by the experiment shown in Figure III.3. The two different peptides (reduced and oxidized) were spiked with a small amount of a peptide standard (S2) to measure the difference in retention times between the standard and the reduced and oxidized peptides. There was a small variation in the retentions times between the reduced and oxidized conformers. However, the difference in retention time between the reduced peptide and the standard was 1.8 min while the difference in retention time between the standard and the oxidized peptide was 1.6 min. Thus, the two reduced and oxidized peptides were eluted from the column without any significant difference in their retention times. The small difference in the retention times represents the error in retention that can be obtained from run to run.

3. *Circular dichroism of reduced and oxidized AcPAK 128-144 peptides*

The state of oxidation of the -SH groups was confirmed peptides using circular dichroism. This method had been used by Matsoukas *et al.* (1984). One advantage using

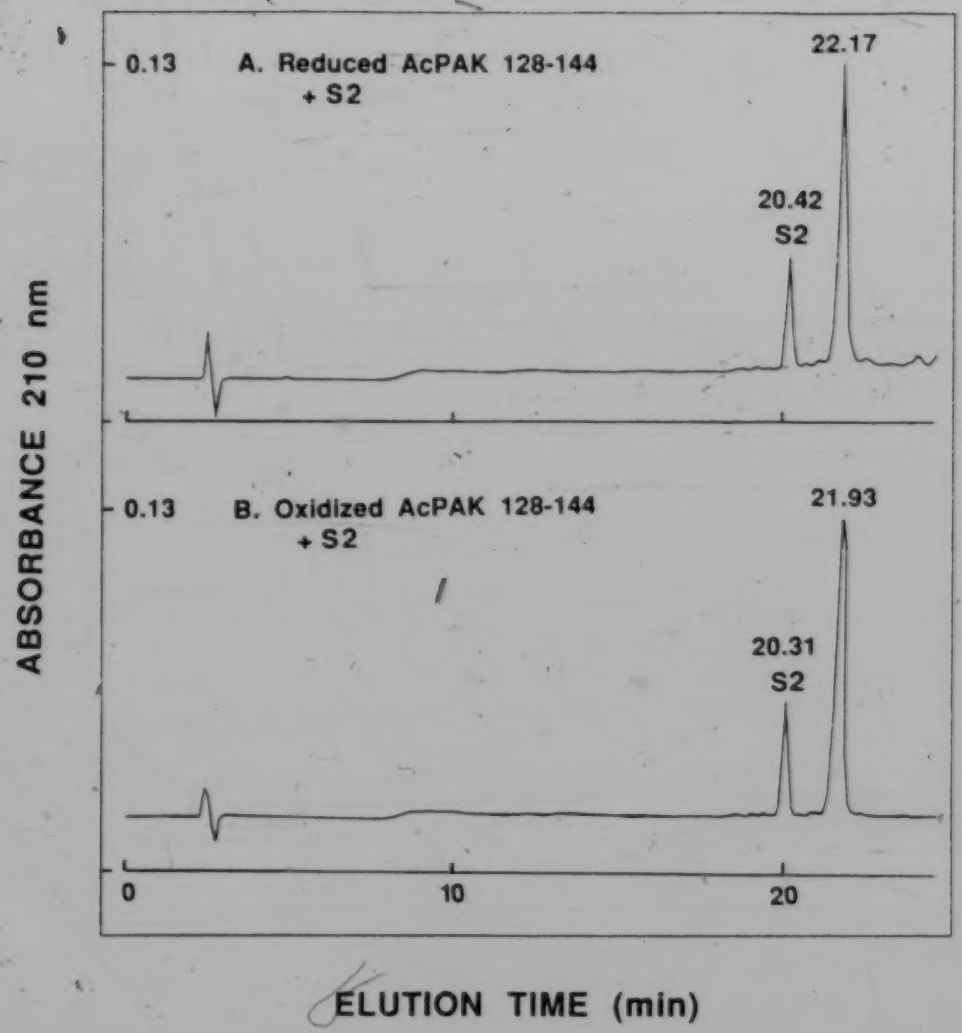


Figure III.3. HPLC profiles of reduced and air-oxidized AcPAK 128-144 peptides chromatographed together with a peptide standard (S2). The chromatographic conditions for these analytical runs have been described in Figure III.2.

this procedure was that the peptides could still be used after the circular dichroism analysis. The results are shown in Figure III.4. The region of the spectrum where useful information was obtained was between 250-350 nm. As shown in Figure III.4A, the difference between the reduced and oxidized peptides was quite distinctive in this region of the spectrum. The oxidized peptides showed a relatively more negative ellipticity than the reduced conformer. Both the air-oxidized and the ferricyanide-oxidized peptides had spectra that overlapped. When an oxidized peptide was mixed with dithiothreitol, a reducing reagent, its spectrum reverted to that of the reduced conformer (Figure III.4B).

4. *Separation of reduced and oxidized peptides*

The resolution of oxidized and reduced conformers may require screening different RPC columns in order to obtain maximum resolution. The packings used for RPC columns vary between different suppliers and the age of these columns affects the resolution of peptides. Of the three different RPC columns that were used (Whatman Partisil, C-8; Synchropak RP-P, C-18; Aquapore RP-300, C-8), it was found that the Aquapore column provided the best separation of the AcPAK peptides. The resolution of a large number of peptides in their reduced and oxidized forms by RPC (linear AB gradient of 1% B/min with a flow-rate of 1 ml/min) were examined and the results are summarized in Table III.1.

A series of 14 peptides in their reduced and oxidized forms was used for this study. The number of amino acid residues within the disulfide loop of the peptides used in this study varied between 4 and 15 residues, and the length of these peptides ranged from 9 to 29 residues. The peptides are numbered 1 to 14 to make it easier to follow in the text. Some of these peptides are closely related analogs varying by single amino acid substitutions (Peptides 13 and 14) or multiple substitutions (Peptides 4 to 7), with others also varying in polypeptide chain length (Peptides 4 and 8; 10 to 14).

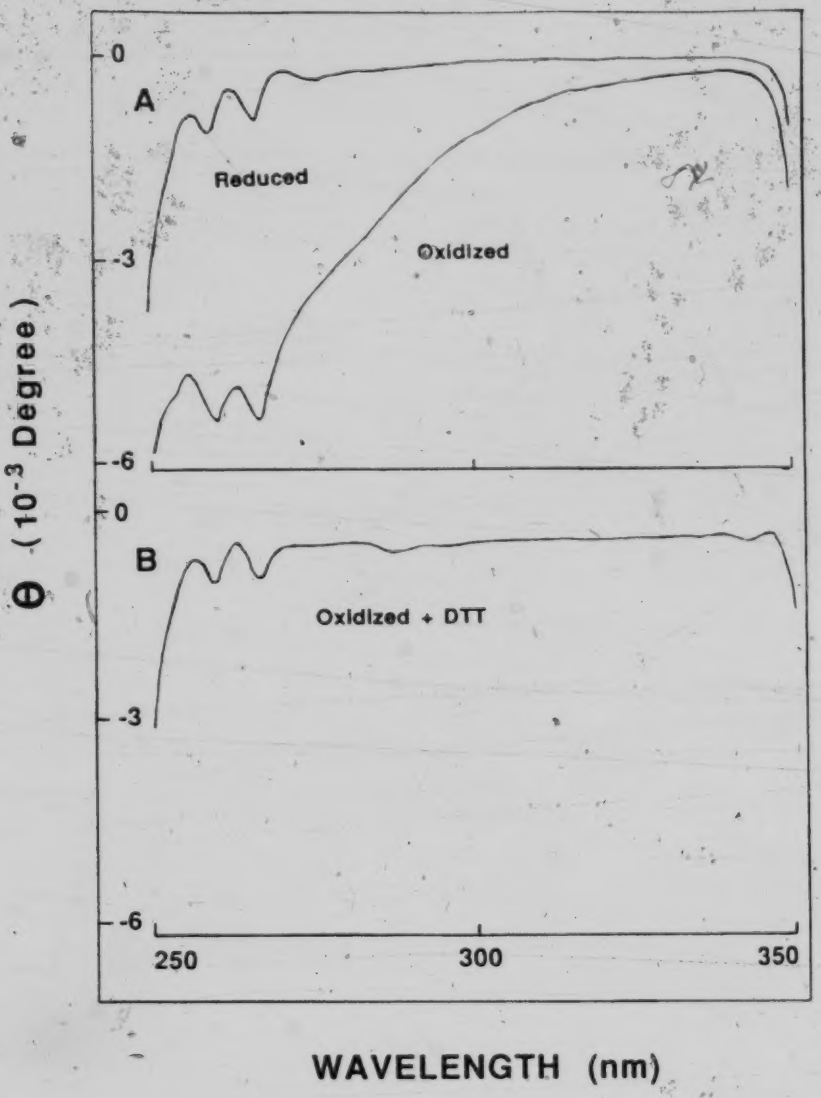


Figure III.4. The circular dichroism spectra of reduced and oxidized AcPAK 128-144. A: a 1.0 mg/ml solution of the peptide was used in each case and the buffer used contained 50 mM phosphate, pH 7.0. The relative ellipticity values (θ) are expressed in millidegrees. B: spectrum of the oxidized peptide after addition of a 1.0 mM dithiothreitol solution.

TABLE III.1 Retention times of reduced and intrachain disulfide-bridged peptides on RPC

Peptide No.	Amino acid sequence[a]	No. of residues within disulfide loop	Retention time (min)[b]		
			(-SH)	(-S-S-)	(ΔRt)
1	NH_2-GIVEC$^{\nabla}$STSIC$^{\nabla}$SLY-NH_2	4	33.22	31.34	1.88
2	desamino-C$^{\nabla}$YFQNC$^{\nabla}$PRG-NH_2	4	26.82	26.82	0.00
3	NH_2-AGC$^{\nabla}$KNFFWKTFTSC$^{\nabla}$-OH	10	35.44	34.44	1.00
4	Ac-KC$^{\nabla}$TSDQDEQFIPKGC$^{\nabla}$SK-OH	12	23.23	21.24	1.99
5	Ac-AC$^{\nabla}$AADQDEQFIPKGC$^{\nabla}$SK-OH	12	25.84	24.09	1.75
6	Ac-AC$^{\nabla}$AAAADEQFIPKGC$^{\nabla}$SK-OH	12	27.56	25.19	2.37
7	Ac-AC$^{\nabla}$AAAAAAQFIPKGC$^{\nabla}$SK-OH	12	31.54	28.29	3.25
8	Ac-ITLTRTAADGLWKC$^{\nabla}$TSDQDEQFIPKGC$^{\nabla}$SK-OH	12	34.89	33.02	1.87
9	Ac-AC$^{\nabla}$KSTQDPMFTPKGC$^{\nabla}$DN-OH	12	24.67	24.67	0.00
10	Ac-C$^{\nabla}$FGGRMDRIGAQSGLGC$^{\nabla}$-NH_2	15	29.80	29.17	0.63
11	Ac-C$^{\nabla}$FGGRMDRIGAQSGLGC$^{\nabla}$NSFRY-OH	15	32.87	32.07	0.80
12	NH_2-SSC$^{\nabla}$FGGRIDR^{o}IGAQSGLGC$^{\nabla}$NSFRY-OH	15	28.82	28.07	0.75
13	NH_2-SLRRSSC$^{\nabla}$FGGRM$^{\blacktriangle}$DRIGAQSGLGC$^{\nabla}$NSFRY-OH	15	29.36	28.73	0.63
14	NH_2-SLRRSSC$^{\nabla}$FGGRM$^{o\blacktriangle}$RRIGAQSGLGC$^{\nabla}$NSFRY-OH	15	29.45	28.07	1.38

a The symbols, ▲ and o denote single amino acid sequence changes between peptides. ∇ denotes position of cysteine or cystine residues; Ac-, acetyl; -OH C-terminal carboxyl group. NH_2, N-terminal amino group

b ΔRt denotes the difference in retention time between reduced (-SH) and (-S-S-) oxidized peptides.

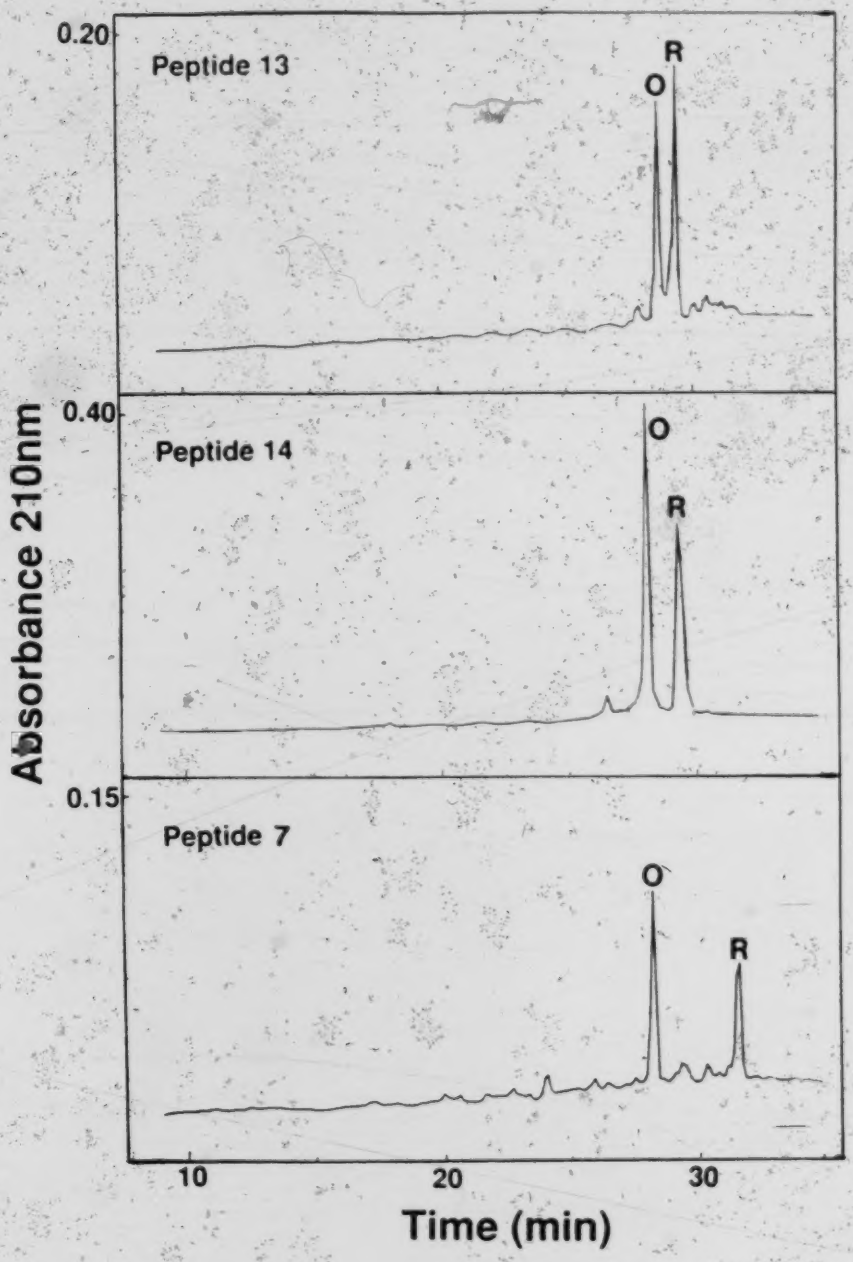


Figure III.5. The separation of three peptides in their reduced and oxidized forms using RPC. Column: Aquapore RP-300 C-8 (220 x 4.6 mm i.d.). The chromatographic conditions have been described in the legend in Figure III.2. The oxidized peptide is denoted by O and the reduced peptide by R.

Representative chromatograms of the separation of the reduced and oxidized forms of three peptides are shown in Figure III.5. The difference in retention time between reduced and oxidized forms of Peptides 7, 13 and 14 was 3.3, 0.6 and 1.4 minute, respectively. In fact, as summarized in Table III.1, the separation of the reduced and oxidized forms of most of these peptides was possible with the exception of Peptides 2 and 9. These two peptides showed no clear separation between the conformers. In addition, no dimeric interchain peptides were observed when a low concentration (0.1 mg/ml) of peptides was used in the air oxidation. Whenever a difference in retention time was observed between the two forms of a peptide, the reduced conformer was always eluted later than the disulfide-bridged peptide. This result suggested that, on formation of the disulfide loop, some residues in the peptide were not as accessible to interact with the stationary phase as they were in the reduced peptide. In other words, the loop formation resulted in a decrease of the hydrophobicity of the peptide binding domain.

5. *RPC of the NEM-modified and oxidized peptides*

Peptides 2 and 9 used in these studies did not show any noticeable separation between the reduced and oxidized forms, and were eluted from the column as a single peak. In order to improve the resolution between the reduced and oxidized conformers, a thiol- specific reagent, N-ethylmaleimide (NEM), was used to modify the reduced peptide. This modification resulted in the formation of a stable covalent linkage between the thiol and NEM, since the thiolate anion attacks one of the double bonded carbon atoms to form N-ethyl succinimidyl cysteine as depicted in Figure III.6. The covalently modified reduced peptide becomes more hydrophobic and is eluted from the column later. Baseline resolution of these two conformers would give us another option for monitoring an oxidation process. The disappearance of the reduced peptide peak with time and the concomitant appearance of the peak corresponding to the oxidized peptide would be

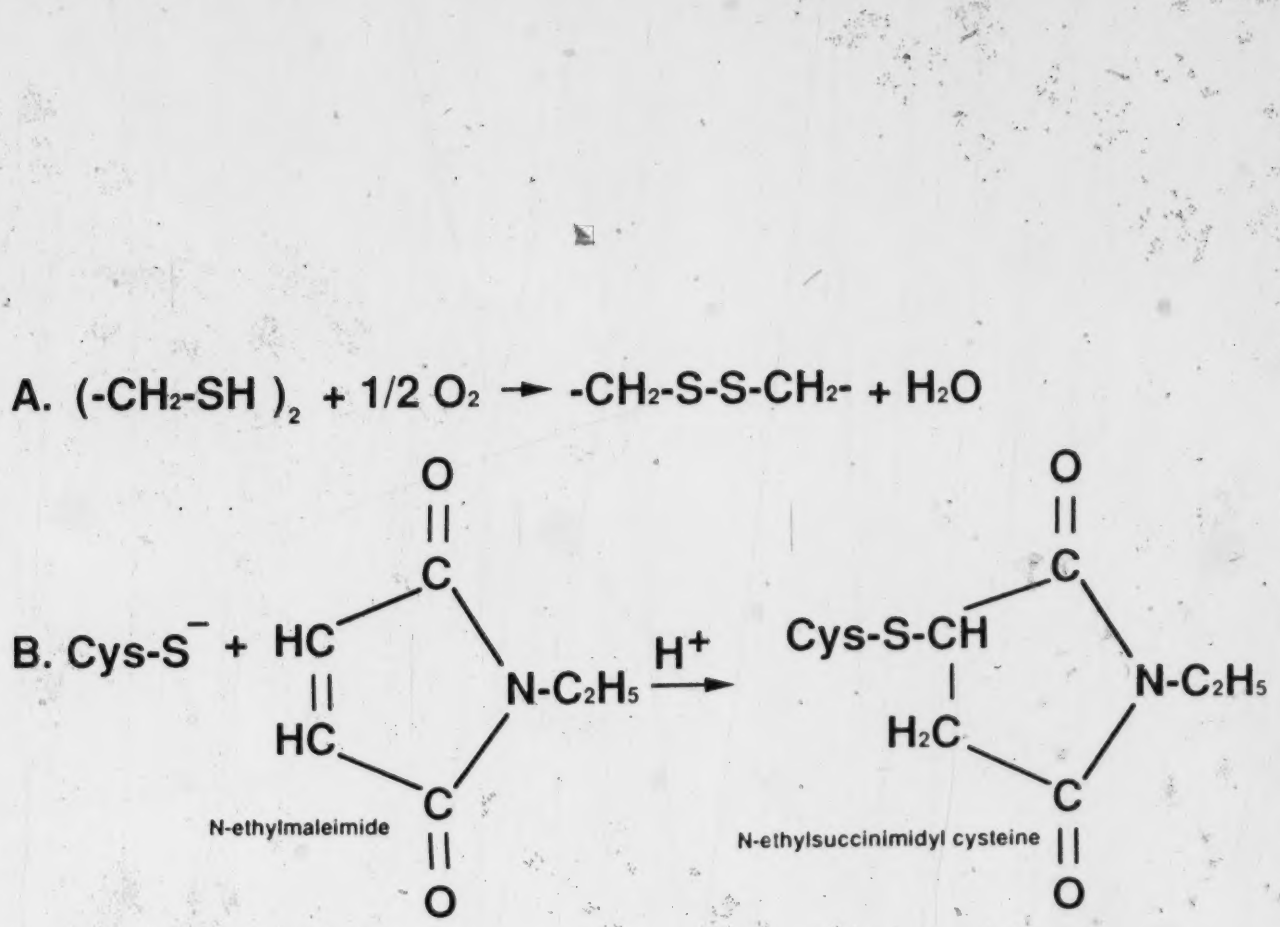


Figure III.6. The air-oxidation reaction of the sulfhydryl groups (A) and the modification reaction of the sulfhydryl groups (B) with N-ethylmaleiimide (NEM) as described by Creighton (1983).

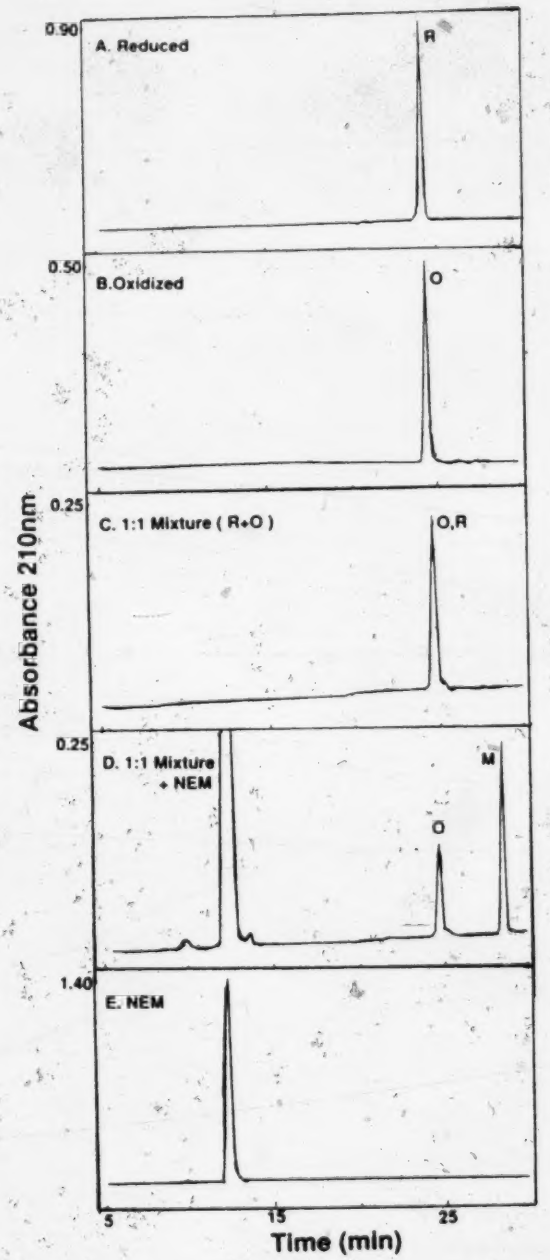


Figure III.7. The separation of reduced and oxidized forms of Peptide 9 following the modification of the reduced peptide with NEM. The NEM-modified peptide is denoted by M. The conditions of chromatography have been described in the legend of Figure III.2.

indicative of the oxidation process. As shown in Figure III.7C, reduced and oxidized Peptide 9 were eluted from the column at 24.8 min and a 1:1 mixture injection resulted in a single peak. When the same mixture was reacted with NEM, it was found that the modified reduced peptide was eluted off the column at 28.6 min. The reagent itself is quite hydrophilic, being eluted from the RPC column after only 12 min. The best resolution of peptides by RPC is usually achieved between 15 - 40% of the organic modifier in the gradient (Hermodson and Mahoney, 1983). Thus, the presence of the excess NEM reagent would not interfere or coincide with most peptides which are eluted off the column later than 15 min.

A more hydrophobic thiol-modifying reagent, N-phenylmaleimide, was also used (data not shown). This particular reagent produced a bigger separation (8 min) between modified and unmodified peptides, compared with NEM. However, the more hydrophobic phenyl rings resulted in the reagent being eluted from the column at around 22 min. This reagent might be coeluted with some peptides and thus, was not the reagent of choice.

The results shown in Figures III.8 and III.9 illustrated the use of RPC to monitor the oxidation of peptides that show no separation between their two conformers. Such a monitoring procedure could be used for any peptides, whether or not they show good separation. At various time points, samples were removed and reacted with the NEM reagent, thus stopping any further oxidation by covalently modifying the free -SH groups of the reduced peptide, denoted by M in Figure III.8. Monitoring the intrachain disulfide bridge formation by RPC allowed simultaneous quantitation of the two conformers, unlike the Ellman titration of free sulfhydryls of the reduced peptides. Although these modified peptides could not be retrieved, only a small amount of peptides are required because of the high sensitivity of UV detection. The modified peptide was also shown to have an enhancement of absorption at 210 nm due to the NEM reagent. The peak height of the modified peptide at the beginning of the oxidation process was 89 mm and the completely oxidized peptide was only 54.5 mm. The injection volumns were 100 µl in both cases.

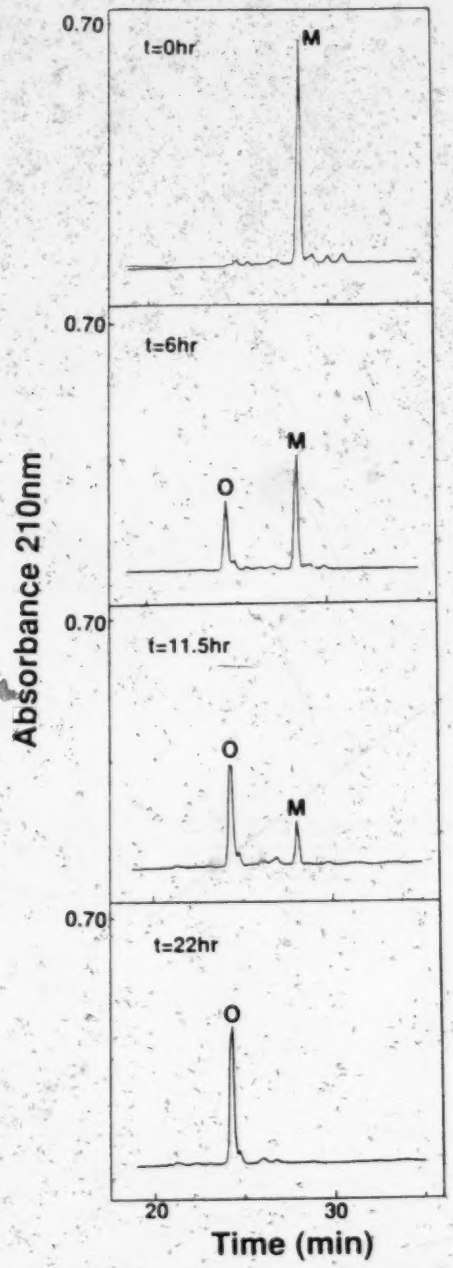


Figure III.8. Monitoring the formation of the intrachain disulfide bridge with time during air-oxidation of Peptide 9 by RPC. The reduced peptide was modified with NEM prior to injection onto the column to increase the separation between oxidized and reduced conformers. The modified peptide is denoted by M and the oxidized peptide by O. The chromatographic conditions have been described in the legend of Figure III.2. Conditions for air-oxidation and chemical modification with NEM are given in the Materials and Methods section.

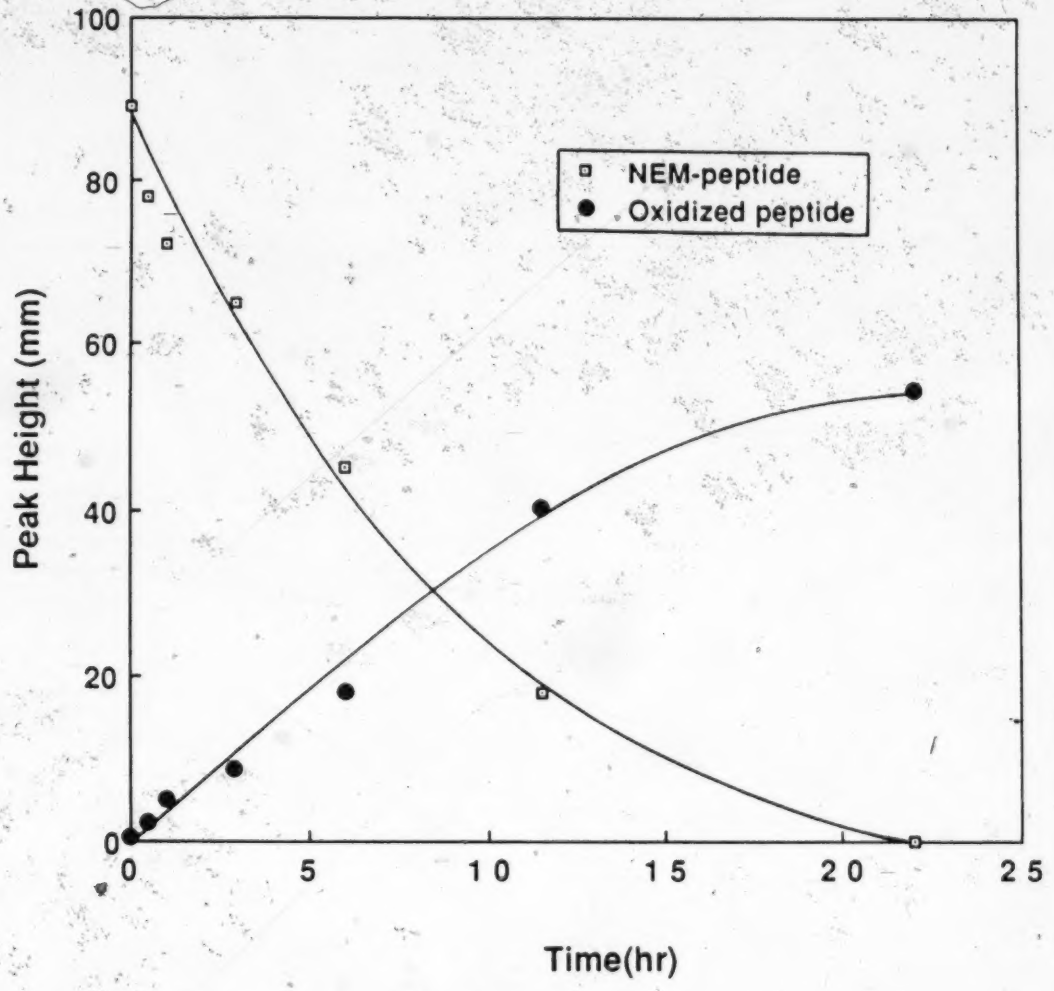


Figure III.9. Quantitative measurements of the reduced and oxidized Peptide 9 during an air-oxidation process. The peak heights of the two conformers obtained from RPC chromatograms (Figure III.8) were used.

This enhancement of absorbance at 210nm would help in detecting any small quantity of reduced peptide left in the air oxidation mixture.

C. Discussion

In these studies, peptides were purified by RPC with a shallow gradient (0.2% B/min) on an analytical column. Purifications on a semi-preparative column (10 mm I.D.) using a gradient of 1% B/min were also carried out. In these instances, multiple runs were made and the peaks of interest were collected as they were eluted off the column. Although these were the only procedures used, there are other methods of purification which have been developed including the novel sample displacement chromatography approach (Hodges *et al.*, 1988; Burke *et al.*, 1988). Different modes of chromatography such as ion-exchange chromatography can also be applied if peptides possess charged amino acid residues. More detailed aspects of the application of HPLC to peptide separation have been reviewed by Mant and Hodges (1990).

A study on the retention behaviour of 14 peptides in their reduced and oxidized forms was carried out. The separation of the two conformers could vary from no resolution at all to as much as 3.3 min. In all instances where resolution was obtained, the reduced conformers were eluted from the column after the oxidized peptide. It has been noted by Creighton (1983) that cystine has a lower solubility in water than cysteine, thus making the disulfide group relatively more hydrophobic than the thiol group. The solubility for cystine in water was 0.01g/100mL, less than 1/1000 that of cysteine. An observation made by Schulze-Gahmen *et al.* (1983) showed that the reduced form of a 16-residue peptide (NH_2-YGCTVGGGGGGGVTCG-OH) was eluted 1-2 min earlier than the oxidized form. The column used by these authors was a Baker C-18 column, 250 x 4.6 mm I.D., 300Å pore-size. The column was run at ambient temperature with a flow rate of 1.0 mL/min and a linear gradient of 2%B/min, where solvent A was 0.1%TFA in water and solvent B was 0.1%TFA in 60% aq. acetonitrile. This observation could be explained

by the increase in hydrophobicity of the peptide due to the presence of cystine versus two cysteine residues. Differences in retention behaviour of the disulfide-bonded peptides, compared to their reduced conformers during RPC, could also be due to differences in conformation of the looped peptides as a result of sequence variability. For example, with the series of peptides (4 to 9), which have 12 amino acid residues between the cysteine residues, the ΔRt between reduced and oxidized conformers ranged from 0 to 3.3 min. Similarly, for peptides with 4 amino acid residues between cysteine residues, the ΔRt for Peptide 1 was 1.9 min while Peptide 2 showed no noticeable difference between the two conformers. In addition, Peptides 13 and 14 differed by only a single amino acid change within the loop (aspartic acid to arginine) and yet the ΔRt between reduced and oxidized conformers varied from 0.6 min for Peptide 13 to 1.4 for Peptide 14. These results suggested that there was no direct correlation between the size of the loop and the differences in ΔRt obtained on RPC.

As we have seen from Table III.1, not all reduced and oxidized peptides were resolved by RPC (Peptides 2 and 9). Some of the peptides tested were only partly resolved (not baseline resolved) even on very good columns, as illustrated by the series of peptides 10 to 13 in Table III.1. Hence, a new method was developed which could resolve the reduced and oxidized peptides. Modification of the free sulfhydryl groups of the cysteine residues with NEM has previously been applied by Lunte and Kissinger (1985) to modify free thiol groups in liver samples for electrochemical detection. These workers were interested in monitoring the presence of free sulfhydryls and cystine groups. In our case, the separation of reduced and oxidized conformers on RPC was the goal. Modification of the reduced peptide resulted in an increase of 3 min in its retention time (i.e., ≅1.5 min per -SH). The reagent reacted only with the reduced peptide (data not shown). The modification resulted in the loss of a small sample of the peptide in the analysis. However, small nanomolar quantities are sufficient for these assays because of the high sensitivity of the ultraviolet detection system. This procedure has an added advantage over the Ellman

method (Ellman, 1959) in that both the reduced and oxidized peptides are monitored simultaneously. In the Ellman method, only the free sulfhydryl groups are monitored and the absence of spectroscopic detection at 412 nm would be interpreted as a completion of the oxidation process. Because the use of this RPC approach, with or without NEM (depending on the baseline resolution of the reduced oxidized conformers), allows one to observe both conformers on a given chromatogram, the oxidation process could be followed by removing samples from an oxidation mixture. The peak area or peak height could be quantitated. An example of this is shown in Figures III.8 and III.9, where the oxidation of Peptide 9 was monitored. The increase in peak height corresponding to the oxidized peptide was followed by a concomitant decrease in the reduced peptide as the oxidation progressed over the time period, illustrated the usefulness of this procedure.

This method of monitoring reduced and oxidized peptides is now routinely used in our laboratory. Such a method of verification of free sulfhydryls may have a wider application than that described for this thesis project. The presence of intrachain disulfide bridges in peptides and proteins is a common feature. Some examples of these are found in peptide hormones such as insulin and vasopressin, and in enzymes such as lysozyme, ribonuclease, serine proteases and other small proteases. The functions of these disulfide bridges are to stabilize the protein (Pace, 1990) and to confer active functional domains. With the technological advances in peptide synthesis, many active fragments of proteins and peptides are being made with their native disulfide bonds or with the addition of disulfide bonds to confer to the fragments a structure similar to that which they exhibit in the native proteins (Atassi *et al.*, 1988). The RPC-NEM approach would provide researchers with a new effective method to monitor the reduced and oxidized conformers of the peptides of interest.

Chapter IV. Immunogenicity and Antigenicity of the C-terminal Disulfide-Bridged Region of *Pseudomonas* Pili

A. Introduction

At the time when this project began, there was the question of the importance of the disulfide bridge located at the C-terminal end of the pilin molecule and its relevance to the immunogenicity of the pilin protein. The cysteine residues of pilin molecules are found to be conserved in all *P. aeruginosa* strains (Johnson *et al.*, 1986; Paranchych, 1989; Pasloske *et al.*, 1988; Sastry *et al.*, 1985). The location of the cysteines within a region of the pilin molecule that is semiconserved could be important from an immunological standpoint. Regions on bacterial and viral components that are immunodominant are often used as a diversion mechanism against the host immune system. The region that is conserved is important for a particular function in the bacterium and its lack of immunogenicity (immunorecessiveness) enhances the survival of the organism in that antibodies would not bind and block the function of the conserved region.

The gonococcus pilus is also a class of NMePhe pilus that is involved in the adhesion of *N. gonorrhoeae* to host cells (Swanson, 1973). The gonococcal pilin has a molecular weight that varies between 17.5 - 21 kilodalton, depending on the strain (Buchanan, 1977). It has a hydrophobic N-terminal region which is homologous to the *P. aeruginosa* pilin (Schoolnik *et al.*, 1984). The C-terminal end of the gonococcal pilin has a disulfide bridge. Cyanogen bromide cleavage of pilin from gonococcal strain MS11 gave rise to a C-terminal disulfide-bridged fragment, CNBr-3, which contained the immunodominant, type-specific antigenic determinant (Schoolnik *et al.*, 1984). Two strain-specific epitopes in regions 121-134 and 135-151 were located within the disulfide loop (Rothbard *et al.*, 1984). Reduction and subsequent alkylation of the intrachain disulfide bond in the CNBr-3 fragment destroyed the antigenicity of this region (Schoolnik *et al.*, 1984). Watts *et al.* (1983) have reacted enzymatically cleaved *Pseudomonas* pilin

fragments with native PAK anti-pilus antiserum and have found that a fragment corresponding to the C-terminal end (residue 121-144) reacted positively in direct ELISA experiments. However, the C-terminal region of the *Pseudomonas* pilin is not the immunodominant region (Watts *et al.*, 1983; Sastry *et al.*, 1985). In contrast with the gonoccocal pilin, modification of the two cysteine residues in the fragment by reduction and carboxymethylation of the disulfide bridge did not abolish the antigenicity of this determinant. It has also been demonstrated in the PAK pilin that the C-terminal region may be part of the pilus-binding domain, and that the C-terminal disulfide bridge is important in maintaining the functionality of this binding domain (Doig *et al.*, 1988; Irvin *et al.*, 1989; Lee *et al.*, 1989; Paranchych *et al.* 1985). We have now investigated the immunogenicity of this region in the synthetic peptides (reduced and oxidized PAK 128-144-OH, PAK(A^{129}) (128-144)-OH) to produce antipeptide antibodies which bind to native PAK pili. The synthesized peptide sequence of the PAK pilin and the corresponding sequence of the disulfide bridge region of PAO pilin are shown in Figure IV.1. The importance of the disulfide bridge to the immunogenicity and antigenicity of this region was further investigated using competition ELISA experiments with both native anti-pilus antiserum and antisera raised against the synthetic peptides. These studies could be important in understanding the role of the disulfide bridge and the significance it might have in developing vaccines against *P. aeruginosa* infections.

B. Results

1. *Peptide synthesis.*

The peptides used in this study, PAK 128-144 and PAK 116-144, were synthesized by the solid-phase procedure described in the Methods and Material Section (Chapter II). The PAK sequences are shown in Figure IV.1. The corresponding region of PAO 128-144 is also shown for comparison purposes as this study relates to both pilin molecules. As noted earlier, the two cysteines form a disulfide bond in the native

```
PAK  116-144  ITLTRTAADGLWKCTSDQDEQFIPKGCSK
PAK  128-144  ............KCTSDQDEQFIPKGCSK
PAO  128-144  ............ACKSTQDPMFTPKGCDN
```

Figure IV.1. Amino acid sequence of the C-terminal region of PAK and PAO pilins. The sequence data were derived from Sastry *et al.* (1985)

molecule. Both the reduced (free sulfhydryl groups on the cysteine residues) and the oxidized (disulfide-bonded) forms of the PAK 128-144 and PAK 116-144 peptides were used in the following experiments.

2. *Determination of the concentration range of BSA-peptide conjugates for ELISA*

The experiments used in this study involved a large number of ELISAs and so a quantitation of the amount of antigens to be coated on the wells of the microplate was first carried out. Four concentrations of the BSA-PAK 116-144 conjugate were tested. Two dilutions of an anti-PAK pilus antiserum, 1:300 and 1:600, were used to perform the quantitation. The results are shown in Figure IV.2. The maximum absorbance was obtained when the wells were coated with an antigen solution of about 10 μg/ml concentration. However, to minimize the amount of antigen to be used while maintaining a high sensitivity in the assays, a solution of 5 μg/ml was used in subsequent experiments. This concentration gave approximately 80-90% of the maximum sensitivity in this assay system.

3. *Antipeptide antisera reactivities with PAK pili*

The antipeptide antisera raised against reduced or oxidized PAK 128-144-OH peptide bound to native PAK pili in direct ELISA experiments. The antipeptide antiserum raised against the reduced and oxidized peptides were designated as 17-R and 17-O, respectively. The antibody titers are tabulated in Table IV.1. The titers were obtained after a subtraction of the A_{405} values of the primed rabbit antisera with values from preimmune antisera. High antibody titers in the range of a 1×10^{-6}-fold dilution were engendered by the peptide-KLH immunogens. The antipeptide antibody titers were similar for both the reduced and oxidized peptides when tested against the corresponding BSA-peptide conjugates. The antipeptide antisera were able to bind to native PAK pili, and titers in the range of 1.5×10^{-5} were recorded. One concern that must be addressed is the possibility

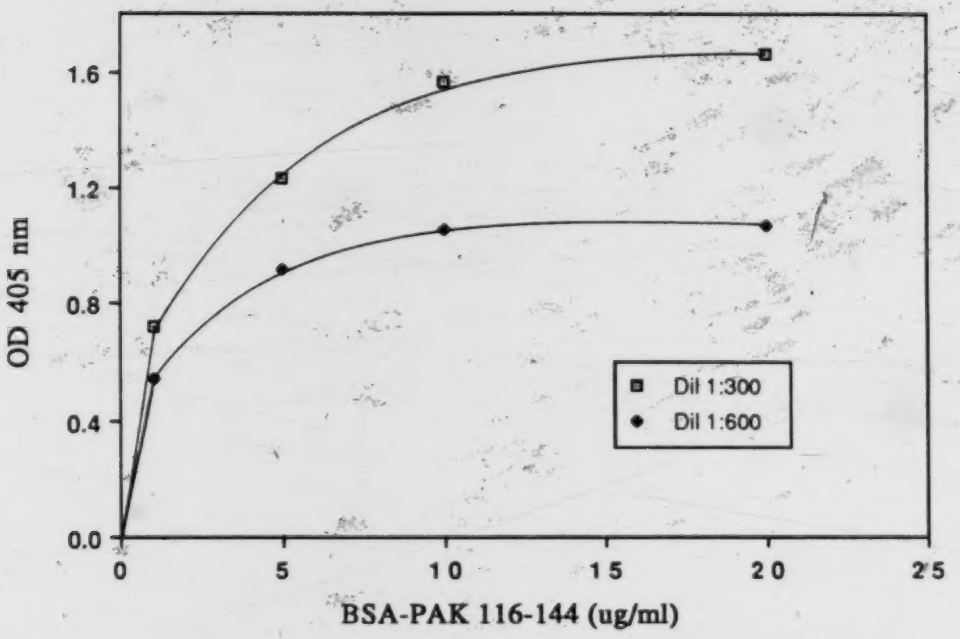


Figure IV.2. Determination of the concentration range of BSA-peptide conjugates to be used for direct ELISA. Two dilutions, 1:300 (▣) and 1:600 (▲) of an anti-PAK pilus antiserum were used with varying concentrations of BSA-PAK116-144 conjugates coated on the wells of the microplate. Absorbance readings at 405nm were taken after a 30 min incubation with the enzyme substrate, *p*-nitrophenylphosphate (1 mg/ml).

TABLE IV.1. End-point titers of antisera raised against reduced and oxidized PAK (128-144)-OH and to PAK (A^{129}) (128-144)-OH peptides

Antisera	End-point Titers[a]		
	Peptide-Conjugate	PAK pili	PAO pili
17-R1	3.5×10^{-6}	$1.0 \pm 1.2 \times 10^{-5}$	$4.15 \pm 3.7 \times 10^{-4}$
17-R2	7.1×10^{-6}	$1.6 \pm 0.4 \times 10^{-5}$	$4.50 \pm 0.3 \times 10^{-4}$
17-01	4.5×10^{-6}	$1.5 \pm 0.7 \times 10^{-5}$	$2.0 \pm 0.8 \times 10^{-5}$
17-02	2.1×10^{-6}	$2.0 \pm 1.2 \times 10^{-5}$	$1.3 \pm 1.6 \times 10^{-4}$
PAK (A^{129})	9.8×10^{-6}	--	--

[a] The end-point titers ± standard deviation (n = 3) determined by direct ELISA experiments for the antisera from five rabbits are shown. R1 and R2 antisera have been raised against reduced PAK (128-144)-OH peptide; while O1 and O2 antisera have been raised against the oxidized PAK (128-144)-OH peptide; PAK A^{129} antiserum has been raised against the cysteine 129-substituted peptide. The end-point was determined as the cut-off at an A_{405} reading of 0.05 absorbance unit above the background value of the control after a 30 min incubation with the subtrate.

that the reduced peptide, when coupled to the carrier protein, does not remain reduced after immunization. One approach would be to carboxamidomethylate the cysteines which would prevent formation of the disulfide bond. However, the presence of the carboxamidomethyl groups would be expected to generate antipeptide antibodies that would also recognize these groups in the peptide and, thus, the antipeptide antibodies would not recognize the native protein. This is strongly supported by the antigen-antibody studies of Hodges *et al.* (1988) which clearly demonstrated that the removal of a single methyl group can result in loss of antibody binding. We felt the only option available was to substitute cysteine-129 with an alanine residue at the N-terminal of the peptide near the site of attachment to the carrier protein. In this way, formation of the disulfide bridge was prevented leaving the C-terminal 15 residues identical to the native protein. Interestingly, the antipeptide antibodies (PAK-A129, Table IV.1) to this sequence bound poorly to the native PAK and did not crossreact with PAO pili, suggesting the importance of the disulfide bond in generating antipeptide antibodies that bind the native proteins or the importance of the side-chain of cysteine-129 in antibody binding. The importance of a disulfide-looped peptide in raising antipeptide antibodies that bind to native protein was illustrated in an early study employing a synthetic peptide corresponding to the looped region (62-84) of lysozyme (Sela *et al.*, 1971). These workers showed that the destruction of the disulfide bridge with performic acid resulted in a loss of binding to native lysozyme.

4. *Crossreactivities of anti-PAK 128-144 antisera with PAO pili*

When the ELISA plates were coated with PAO pili, it was found that the 17-R and 17-O antisera were able to bind and crossreact with the heterologous pili. However, only antiserum 17-01 (Table IV.1) gave an end-point titer with PAO pili similar to PAK pili. The other three antisera titers with PAO pili were at least 10-fold lower compared to PAK pili. From the sequence data (Figure IV.1), it can be seen that the amino acid residues in the C-terminal end of the PAK and PAO pilin proteins are only semi-conserved. From the

primary amino acid sequences, surface profile predictions based on the combination of hydrophilicity, mobility and accessibility parameters (Parker *et al.*, 1986) were carried out and it was shown that the C-terminal regions of both PAK and PAO pilin are surface exposed regions (Chapter V, Figure V.1). This C-terminal region of PAO pilin also contains two cysteine residues which can form a disulfide bridge in the native molecule. The results of the direct ELISA experiments showed that the anti-peptide antisera raised against the oxidized peptide were able to bind better to PAO pili than those raised against the reduced peptide, PAK (128-144)-OH. This was also shown by the competitive ELISA experiments (see below). This was not the case with PAK pili, where antisera to reduced or oxidized peptide had similar binding activities. These crossreactivities were also observed in immunoblot assays.

5. *Immunoblot assays*

The binding of anti-peptide antibodies to pili was checked using immunoblot assays (Figure IV.3). Purified PAK and PAO pili (lanes 1 and 3, respectively) and also pili from the lysate fractions of whole cell *P. aeruginosa* strains PAK and PAO (lanes 2 and 4, respectively) (see Materials and Methods) were used. A faint band appearring on the lefthand side of lane 1 in each of the panels is a low molecular weight prestained colored marker. As shown in panel *a* (Figure IV.3), anti-PAK pilus antiserum bound to homologous pili and also crossreacted with heterologous PAO pili. The lysate fractions from PAK and PAO contained some other bands which crossreacted with the anti-PAK pilus antiserum. Anti-PAO pilus antiserum bound to homologous pili and also crossreacted with PAK pili (panel *d*, Figure IV.3). The pilin bands form the lysate fractions from PAK and PAO whole cells appeared as doublets and the cause for this is not known. Both anti-peptide antisera raised against reduced (17-R) and oxidized (17-O) peptides reacted with purified pili and pili from the whole cell PAK preparation. It was also seen that the two different antisera, 17-R and 17-O, crossreacted with purified PAO pili (panels *b*, *c*, *e* and

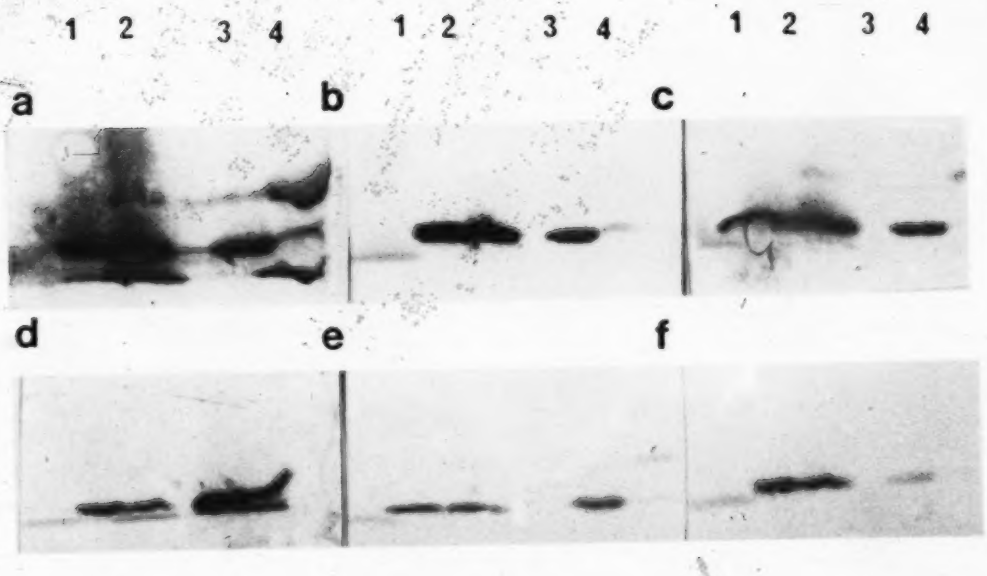


Figure IV.3. Immunoblot assays demonstrating the interaction between purified pili and whole cell pili from PAK and PAO strains with all four antipeptide antisera 17-R1, 17-R2, 17-O1 and 17-O2 (Panels b, c, e and f, respectively). Lane 1 is purified PAK pili; lane 2 is solubilized membrane of PAK whole cell; lane 3 is purified PAO pili; and lane 4 is solubilized membrane of PAO whole cell. Control experiments using antisera raised against native PAK and PAO pili are shown in panels a and d, respectively.

f). This interaction between anti-PAK 128-144 antibodies and PAO pili was not as strong as that observed with the PAK pili, even though an approximately equal amount of material was loaded onto the gel. However, antisera 17-O1 showed equally intense pilin bands with PAK or PAO pili (panel *e*), consistent with the ELISA data of Table IV.1. Interaction of the antipeptide antisera with PAO pili from whole cell only gave faint bands. This could be due to smaller quantities of pili produced per PAO cell, or to a combination of a reduced amount of pili and the weaker interactions between antisera and pili. In addition to the interactions with the PAO pilin molecules, some crossreactivities were observed between the antipeptide antisera with a high molecular weight band from the PAO lysate fraction (especially in panels *c* and *e*).

6. *The relative affinities of antipeptide antibodies to PAK pili*

The relative affinities of the antibodies raised against reduced and oxidized peptides for the PAK pili were determined using competitive ELISA experiments. Protein-A purified IgG (1.0 nM) from each serum (17-R1, 17-R2, 17-O1 and 17-O2) were incubated with a serially diluted solution of reduced and oxidized AcPAK 128-144-OH peptides. The results are shown in Figures IV.4 and IV.5. In each instance, where either reduced or oxidized peptide was used to compete for binding to IgG, there was no significant difference in the ID_{50} (inhibition dose which gave a fifty percent reduction in binding of the peptides for either IgG raised against the reduced or oxidized 17-residue peptide). In the case of reduced peptides, incubations were carried out in the presence of a small concentration of β-mercaptoethanol to keep them in a reduced state. The antibodies that were raised against the oxidized peptide were also able to recognize the reduced peptide and vice-versa. This suggested that the native conformation with the disulfide chain was not critical in the antigenicity of this region. This was illustrated again when native pili were used as the competing antigen with either IgG raised against the reduced or oxidized peptide, as shown in Figure IV.6. When the 17-R or 17-0 IgG was preincubated with

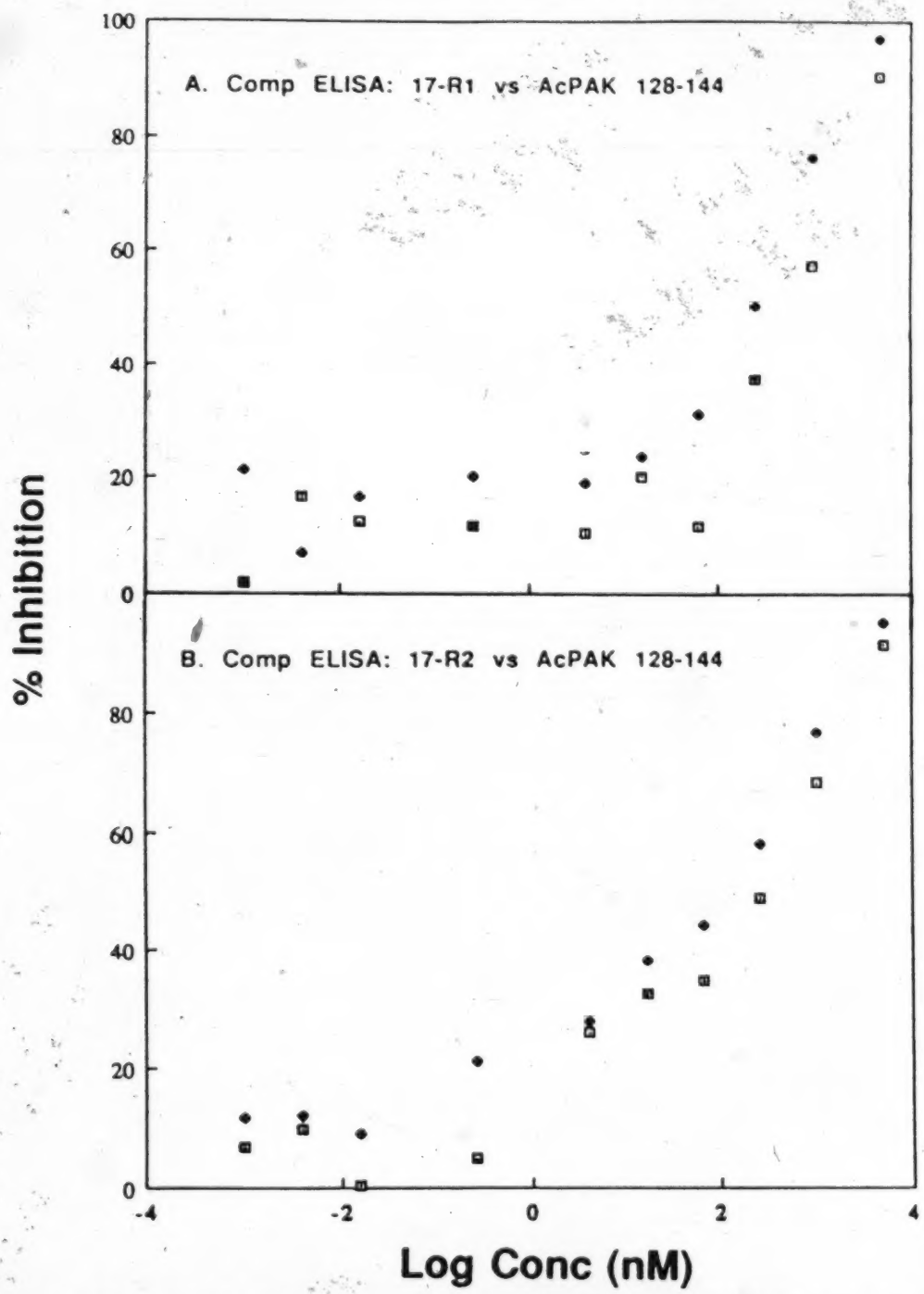


Figure IV.4. Competitive ELISA with IgG (10nM) of 17-R1 and 17-R2. The wells on the microplate were coated with a 5 ug/ml PAK pili solution. The competing antigens used in these assays were AcPAK 128-144 oxidized peptides (▲) and reduced peptides (⊡). The abilities of the peptides to bind and compete at a given concentration is represented as a percentage of the absorbance in the absence of competing antigen.

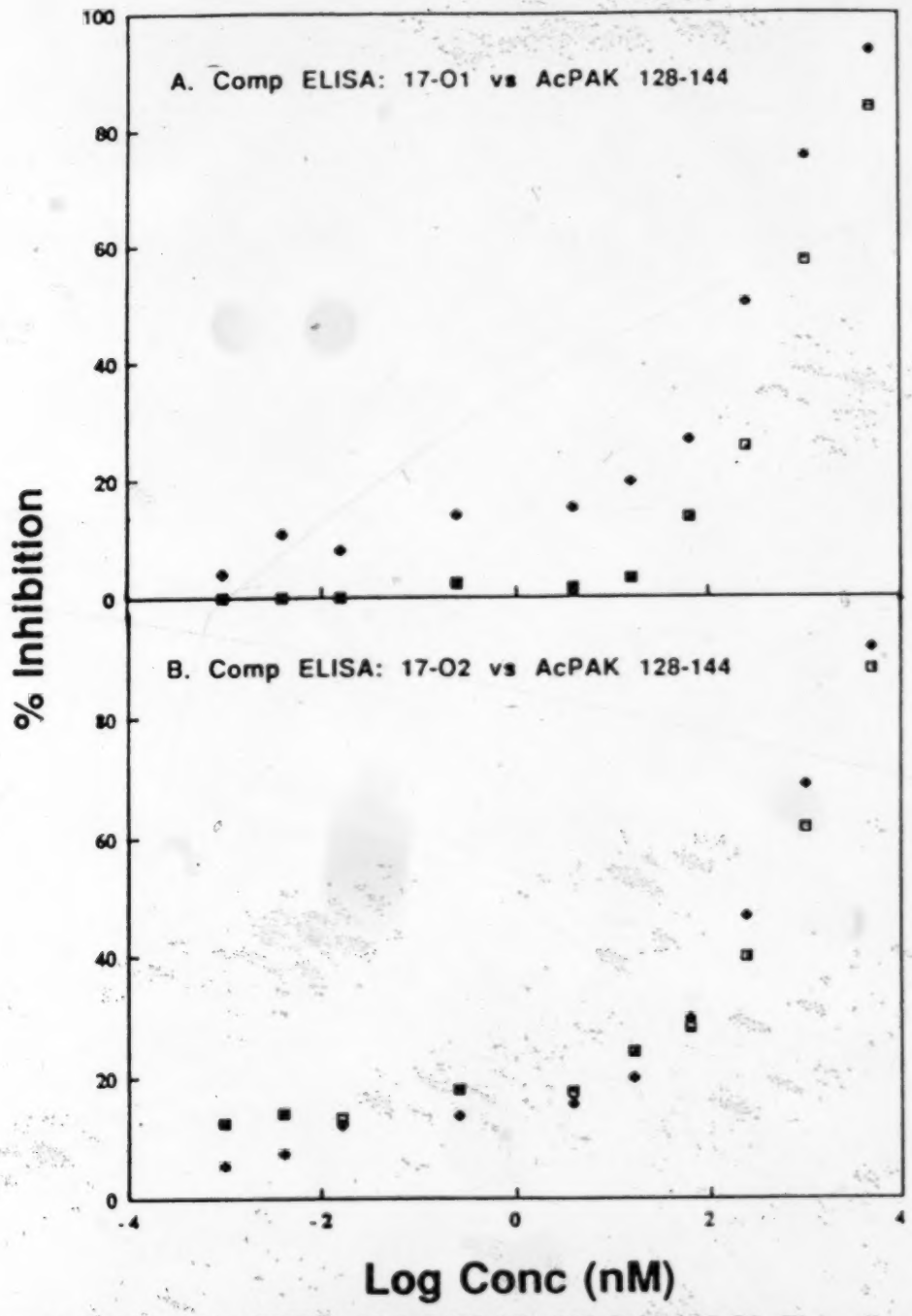


Figure IV.5. Competitive ELISA with IgG (10nM) of 17-O1 and 17-O2. The wells on the microplate were coated with a 5 ug/ml PAK pili solution. The competing antigens used in these assays were AcPAK 128-144 oxidized peptides (▲) and reduced peptides (⧈). The abilities of the peptides to bind and compete at a given concentration is represented as a percentage of the absorbance in the absence of competing antigen.

native PAK pili, the competitive ELISA experiments showed that all four sets of data points could be fitted by a single line, illustrating the similarities in the antibody interactions of the two different antibodies with PAK pili. Another set of experiments was carried out with papain-digested IgG, i.e., the Fab fragments, to show that the bivalency of IgG did not affect the results (Figure IV.7A and IV.7B). Although the inhibition patterns using Fab fragments were similar to those obtained with IgG (in that both reduced and oxidized peptides had similar affinities for the antigen binding sites), a much higher concentration (about 26-fold) of the competing peptides were required in the assays with IgG to give rise to a 50% inhibition.

7. *The binding of an anti-PAK pilus antiserum to PAK 128-144 and PAK 116-144*

The binding of an anti-PAK pilus antiserum to PAK 128-144 and PAK 116-144 was assayed and the results compared. The results are shown in Figures IV.8 and IV.9. As indicated by the intensities of the absorbance at 405 nm, the antiserum bound better to the oxidized peptides than the reduced peptides under the conditions used in the direct ELISA. The antibodies also bound better to the longer peptide than the shorter one. These results indicated that the C-terminal region is immunogenic.

8. *The binding of anti-PAK pilus antiserum to the C-terminal peptide*

The binding of native PAK anti-pilus antiserum to the C-terminal AcPAK 128-144 peptide was also studied using competition ELISA experiments. In this case, the wells of the ELISA plates were coated with the reduced or oxidized peptide-conjugate. The peptide-conjugate was used instead of native PAK pili because the C-terminal region is not the immunodominant region and the percentage of antibodies in the native anti-pilus antiserum which bind to this region is less than 10 percent. Any changes in the absorbance readings due to the inhibition by the peptides would be small and, hence, if pili were used to coat the

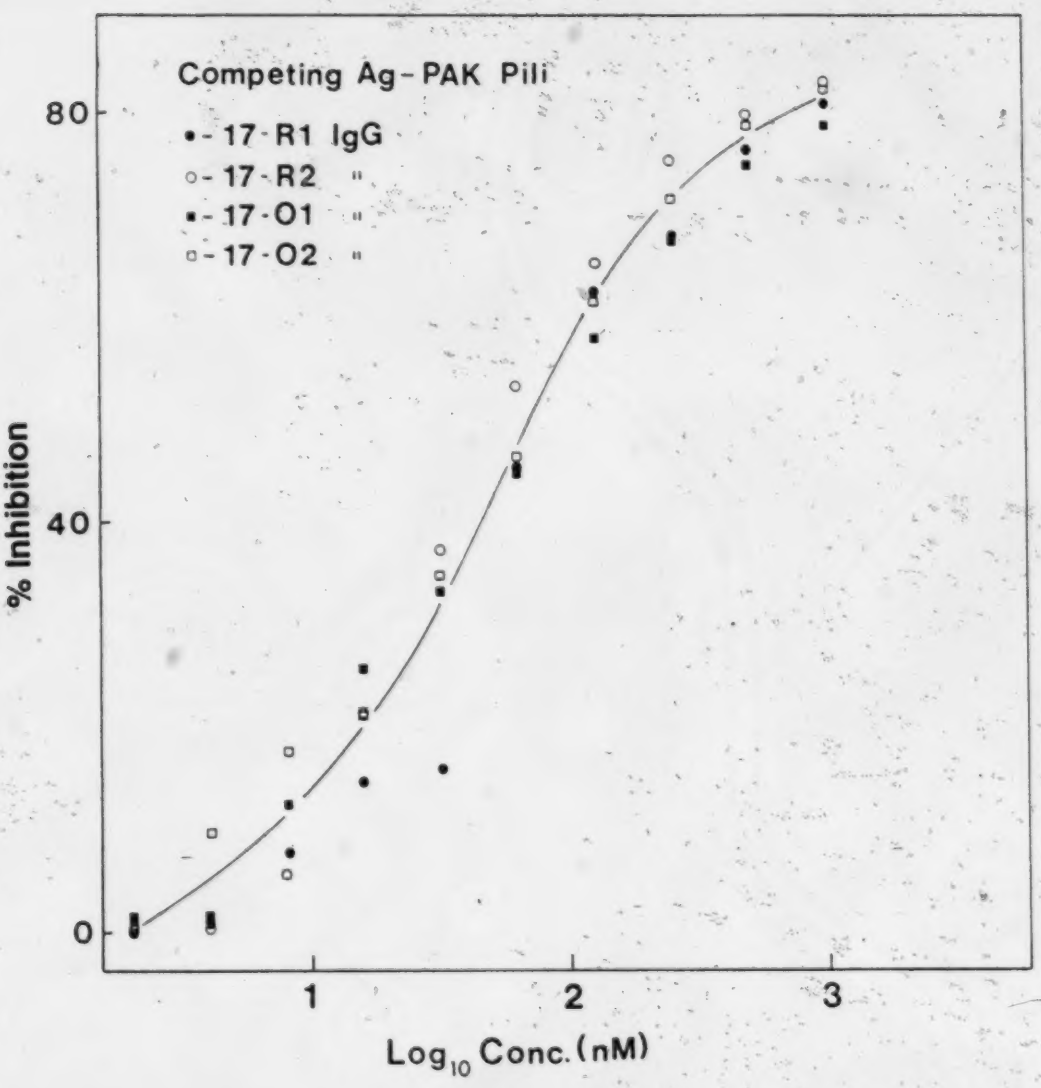


Figure IV.6. Competitive ELISA with native PAK pili. The wells were coated with a 5 ug/ml PAK pili solution and four antipeptide antibodies (1.0 nM) were assayed; ●, 17-R1; ○,17-R2; ■, 17-O1; and ▫, 17-O2. A single line was able to fit all four sets of data points, illustrating the similarities in their interactions with PAK pili.

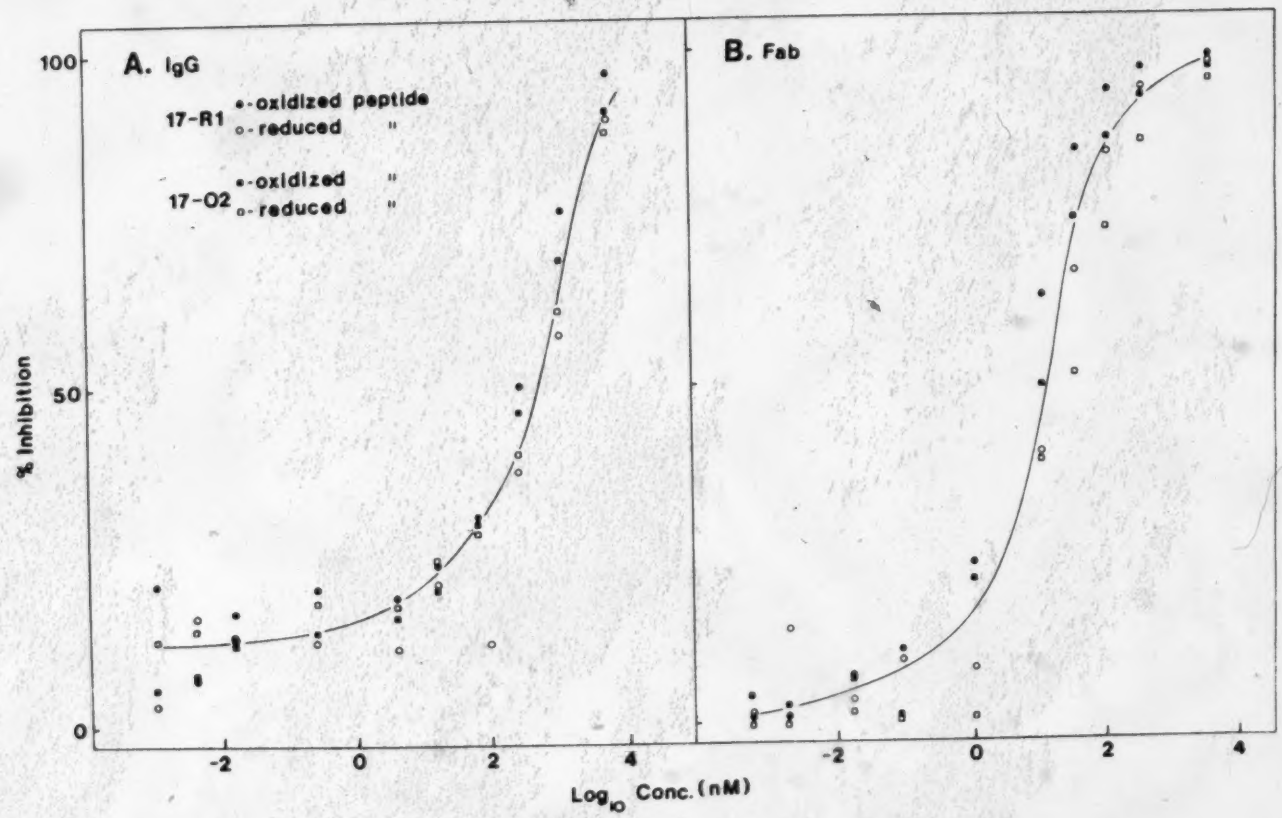


Figure IV.7. Comparison between IgG (panel A) and its Fab fragments (panel B) in competitive ELISA. Two antipeptide antibodies, 17-R1 and 17-O2, and their Fab derivatives are shown. The wells were coated with 5 ug/ml PAK pili and competed with varying concentrations of reduced and oxidized AcPAK 128-144 peptides. The affinities of these peptides are represented by the percent inhibition.

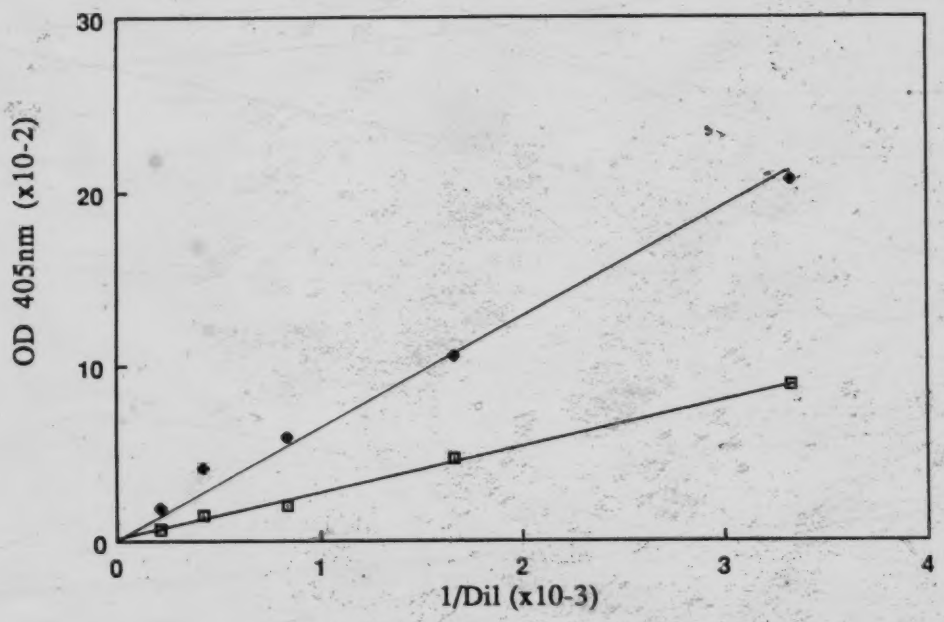


Figure IV.8. The binding of an anti-PAK pilus antiserum to the BSA-peptide conjugates of PAK 128-144. Both the reduced (⊡)and oxidized (▲) forms of the AcPAK 128-144 peptide were assayed. The absorbance readings at 405nm were taken after a 30 min incubation with the enzyme substrate.

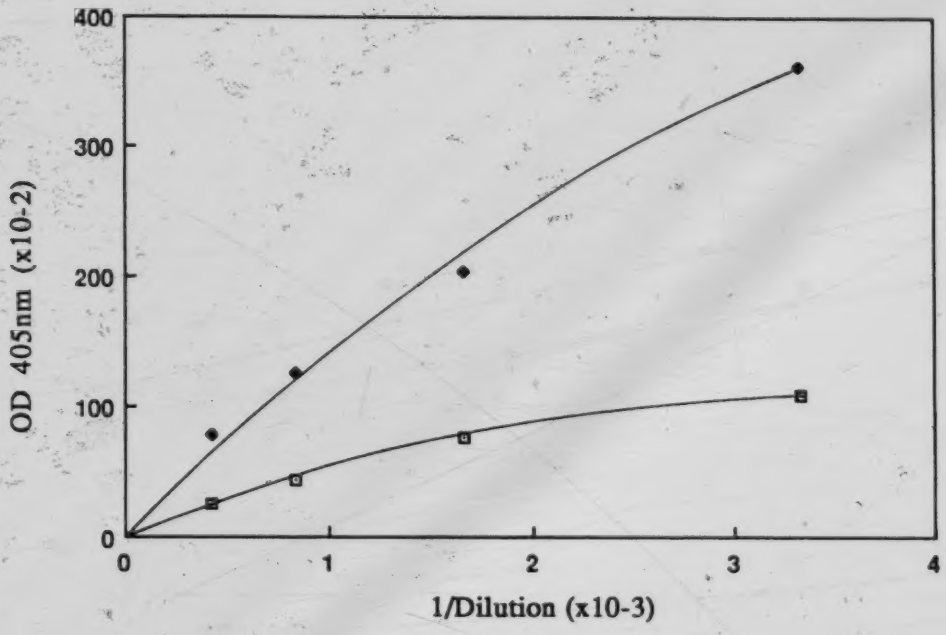


Figure IV.9. The binding of an anti-PAK pilus antiserum to the BSA-peptide conjugates of PAK 116-144. Both the reduced (⊡)and oxidized (▲) forms of the AcPAK 116-144 peptide were assayed. The absorbance readings at 405nm were taken after a 30 min incubation with the enzyme substrate.

wells, these small changes would be difficult to detect in the presence of all the other epitopes on the pili which would be bound by other antibodies in the polyclonal serum. Native antiserum (1:8000 final concentration) was pre-incubated with oxidized or reduced peptide before addition to the peptide-conjugates. The incubation with the reduced peptide and coating of the wells with reduced peptide-conjugate were carried out in the presence of β-mercaptoethanol. The results are shown in Figure IV.10A and IV.10B. When the wells were coated with oxidized peptide-conjugate, competition with reduced or oxidized peptide showed similar inhibition patterns with almost identical ID_{50} values. The converse experiment, with wells coated with reduced peptide-conjugates, gave similar results with reduced and oxidized peptides competing equally well for binding to antibodies from native PAK anti-pilus antiserum.

9. *Crossreactivities of anti-PAK peptide antisera with PAO pili*

An interesting result was obtained when PAO pili were used as the competing antigen in these competitive ELISA experiments with IgG molecules and the wells were coated with PAK pili. Earlier results with the direct ELISA and immunoblot experiments have shown that all four of the antipeptide antisera raised against either reduced or oxidized peptide were able to bind to PAO pili. As shown in Figure IV.11, only one of the four antisera (17-O1), was saturated and competed out by the PAO pili. The other three antipeptide antisera showed little or no preference for binding to PAO pili. With the 17-01 IgG, the ID_{50} when PAO pili was used as the competitor was 4.36 fold less compared with PAK pili. From the amino acid sequence of the disulfide bridge region, both PAK and PAO show some sequence homology (nine out of the seventeen residues). There is a stretch of five out of six residues which are conserved in one region. It is possible that, on binding to the ELISA wells and to the nitrocellulose paper, denaturation of the pilin subunits may enable all four anti-peptide antisera to recognize and

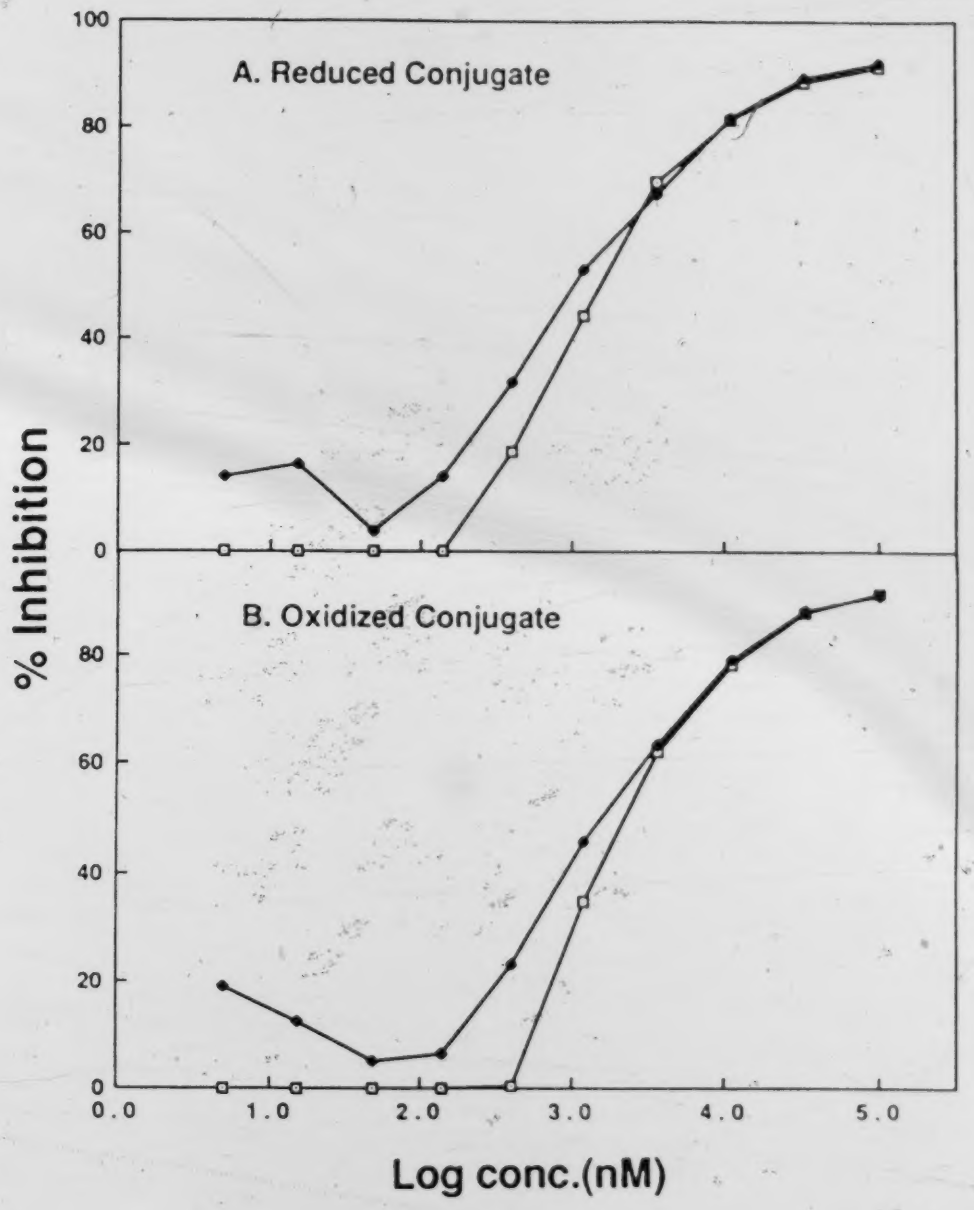


Figure IV.10. Competitive ELISA with anti-PAK pilus antiserum. The wells were coated with a 5 ug/ml solution of BSA-PAK 128-144 conjugate in either the reduced (A) or oxidized (B) form. The reduced conjugate was incubated with a small amount of reducing reagent to keep it in the reduced state. The concentration of the native antiserum used was a 1:8000 dilution. The conjugates were competed with reduced (▫) and oxidized (♦) AcPAK 128-144 peptides.

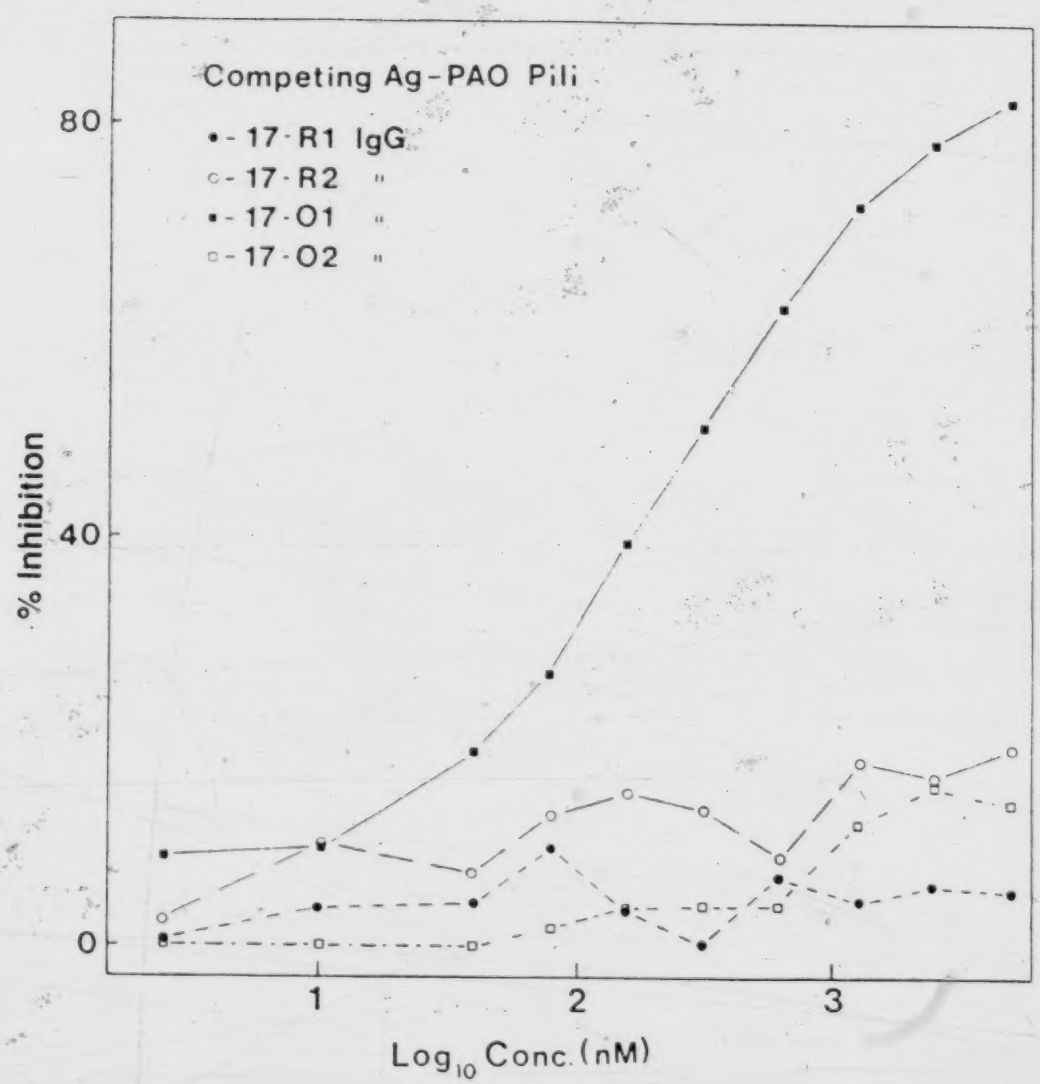


Figure IV.11. Competitive ELISA with native PAO pili. The wells were coated with a 5 ug/ml PAK pili solution and four antipeptide antibodies (1.0 nM) were assayed; ●, 17-R1; ○ ,17-R2; ■, 17-O1; and □ , 17-O2.

bind to this conserved region with affinities in the ELISA and immunoblot experiments. In a competitive ELISA test, where the PAO pili are in solution, the pili are in their native state and only the 17-01 IgG which recognizes this native conformation would bind to give rise to the inhibition that was observed. The observation that only one of the two antisera raised against oxidized peptide (17-01 and not 17-02) bound to PAO pili could have been attributed to different specificities of those antibodies prepared in different rabbits. Although all four antisera reacted equally well with the PAK pili in the competitive ELISA in Figure IV.6, it was interesting that only the 17-01 IgG bound well enough to PAO pili to give a binding curve. The kinds of interactions observed in the binding to the C-terminal region of the PAK pili were equally strong, as shown by the similarities in the binding curves in the competitive ELISA, but the abilities to bind to PAO pili were different. Even though both the 17-01 and 17-02 antisera were raised against the same oxidized peptide, only one of them recognized and bound PAO pili. As noted earlier in Figure VI.1, there is a stretch of five out of six amino acids which is conserved in both PAK and PAO pilins. The 17-01 IgG may be recognizing this epitope and the 17-02 may recognize a separate epitope.

C. Discussion

The application of synthetic peptides corresponding to the C-terminal disulfide-bridged region of the *P. aeruginosa* PAK pilin has allowed a better understanding of the immunogenicity and antigenicity of this region. Anti-PAK pilus antiserum was shown to bind synthetic peptides corresponding to residues 128-144 and 116-144 of the PAK pilin (Figure IV.8 and IV.9). This suggested that this region of the PAK pilin is immunogenic. These results were in agreement with the findings of Sastry and Paranchych (1985) and Watts *et al.* (1983). In their studies, a fragment of the PAK pilin (121-144) was found to be weakly immunogenic and reduction and carboxymethylation of the sulfhydryl groups did not affect the antigenicity of the peptide. The anti-PAK pilus antiserum was found to bind better to the 29-mer than its shorter 17-mer version. This may be due to the presence of a fraction of antibodies that could bind to the additional residues prior to the disulfide loop region, or perhaps the longer peptide gave rise to conformational epitopes akin to those found in the native pilin, thus enabling better binding to antibodies in the serum. The data in Figures IV.8 and IV.9 also suggested that the oxidized conformers (PAK 128-144 and 116-144) showed better binding to the anti-PAK pilus antiserum than the corresponding reduced conformers. The formation of the disulfide loop could have resulted in a conformation in the peptide which resembles its conformation in the native pilin. Although the competitive ELISA Figure IV.10 showed that the anti-pilus antibodies bound well to both reduced and oxidized AcPAK 128-144-OH, there may be a slight preference for the oxidized over the reduced conformer. From Figure IV.3, an estimation of the ID_{50} revealed that with the reduced PAK 128-144 conjugate immobilized onto the solid phase, the ID_{50} for the oxidized AcPAK128-144 peptide was 1.0 nM *versus* an ID_{50} of 1.6 nM for the reduced conformer. With the oxidized PAK 128-144 conjugate immobilized on the microplate, the ID_{50} for the oxidized AcPAK 128-144 peptide was 1.6 nM *versus* an ID_{50} of 2.2 nM for the reduced AcPAK 1289-144 peptide. The ability of the reduced peptide to bind to the anti-PAK pilus antibodies, whose epitope has a disulfide

bridge, may be attributed to the ability of the peptide to fold into the pocket of the antigen binding site. It is also possible that the peptide has an inherent ability to fold into a conformation akin to the native protein. The disulfide bond locks the conformation into a stable configuration which could result in a higher binding affinity for the antibodies.

When the synthetic PAK 128-144 peptides were used as immunogens, it was shown that antipeptide antisera to both reduced (17-R) and oxidized (17-O) conformers bound very well to the PAK pili in direct ELISA and immunoblot assays (Table IV.1 and Figure IV.3). Although this region of the pilin molecule is immunorecessive, the use of synthetic peptides have helped overcome this problem. High antibody titers were obtained and these antibodies bound to this region on the native protein. Furthermore, there is a conserved epitope in this region of the *P. aeruginosa* pilin molecule that allowed crossreactivities between the 17-R and 17-O antipeptide antisera with the PAO pili. The integrity of the disulfide bridge may be important in the generation of crossreacting anti-PAK 128-144 antibodies since the 17-O1 antibodies were the only ones of the four antisera that could effectively compete for binding to PAO pili in indirect ELISA (Figure IV.11). The titers of these other three antisera (17-R1, 17-R2 and 17-O2) were also 10-fold lower than the 17-O1 when assayed against PAO pili in direct ELISA (Table IV.3). This was also illustrated by the immunoblot assays (Figure IV.3). The PAO pilin band lighted up by the 17-O1 antiserum was as intense as those of the PAK pilin bands. The other three antisera gave rise to a PAO pilin band that was much fainter. Thus, the epitopes that are recognized by the 17-O1 antibodies may be different from the epitopes recognized by the 17-R1, 17-R2 and the 17-O2 antibodies. Although the 17-O2 antiserum was also raised against the oxidized PAK 128-144 peptides, it did not bind to PAO pili as well as the 17-O1 antiserum. Obviously, the epitopes of the 17-O1 and 17-O2 were significantly different to account for the different affinities. Recognition of the oxidized peptides by different populations of B-lymphocytes in the two different rabbits that were used have led to

production of antibodies that have subtle differences in their epitopes; these differences gave rise to strain-specific and crossreactive antibodies.

The fact that the disulfide bridge is not critical to the antigenicity of this C-terminal region of *P. aeruginosa* seems to be in contrast to the results obtained with gonococcal pilin (Schoolnik *et al.*, 1983). The results of the present studies and those of Watts *et al.* (1983) showed that the antigenicity of the disulfide loop region of *Pseudomonas* pilin was not destroyed by the disruption of the disulfide bond. The disulfide loop region in the gonococcal pilin, which has 30 residues within the loop (Schoolnik *et al.*, 1984) is larger than the *Pseudomonas* pilin, which only contains 12 residues within the loop. Schoolnik *et al.* (1983) reported that the reduction and carboxymethylation of the cysteines in the loop region of gonococcal pilin resulted in the destruction of strain-specific immunodominant epitopes. There were no detailed data presented in their report. These workers proposed that this intrachain disulfide bond conferred to the loop region a conformational epitope. However, subsequent studies by these workers (Rothbard *et al.*, 1984) revealed two strain-specific antigenic determinants within this loop region (121-134 and 135-151). Antipeptide antisera against these two regions bound to the gonococcal pili and inhibited adhesion of *N. gonorrhoea* organisms to mammalian cells (Rothbard *et al.*, 1985). In addition, reactivities of monoclonal antibodies to the highly variable region in the disulfide loop of the gonococcal pilin block pilus binding to epithelial cells, suggesting that the loop region may contribute to pilus-host cell adhesion (Nicholson *et al.*, 1987). In a comparative study of the NMePhe pilin from different genera by Dalrymple and Mattick (1987), it was observed that the C-terminal region of pilin from *N. gonorrhoea*, *P. aeruginosa* and *M. bovis* contains two cysteines which form a putative disulfide bond. In *B. nodosus*, the location of the cysteine residues are in the central region of of the pilin molecule. The conservation of cysteine residues and the formation of a putative disulfide loop suggest that this loop region could play an important role in pili function.

The studies here show that antibodies in the polyclonal sera raised against PAK pili are able to recognize and bind to the C-terminal 17-mer peptide in the presence or absence of an intra-chain disulfide bridge. This suggests that the antigenic determinants in this region of the pilin molecule are mostly linear epitopes when native pili is used as the immunogen. Thus, this disulfide bridge is not critical in the antigenicity of this region unlike the case of the gonoccocal pilin. Although the presence of an intact disulfide bridge was not important in the antigenicity of the *Pseudomonas* pilin, it was found that only one of the antipeptide antisera which was raised against the oxidized peptide was able to bind and cross-reacted well enough with PAO pili to give good inhibition curves in an indirect ELISA experiment. Hence, there may be other structural features in the native oxidized state which allowed us to raise antibodies that cross-reacted well with other *Pseudomonas* strains. This cross-reactivity would be important in looking for a suitable vaccine candidate for *Pseudomonas aeruginosa.*

Chapter V. Mapping the Surface Regions of PAK and PAO Pilins: Crossreactive and Strain-Specific Antibodies

A. Introduction

Bacterial infections are multistep processess (Smith, 1984). They involve the adherence and colonization of the organisms on the epithelial surfaces of the host, the invasion into the mucosal layer and dissemination of the organisms in the tissues of the host. The prevention of the first step to an infection is the key to producing an effective vaccine. Hence, the understanding of the adherence processes of *P. aeruginosa* to epithelial cells could yield vital information towards the achievement of producing an effective *P. aeruginosa* vaccine. *P. aeruginosa* appears to be capable of employing two adhesins when adhering to epithelial cell surfaces (McEachran and Irvin, 1985; Ramphal and Pier, 1985; Ramphal *et al.*, 1987). The adhesins for nonmucoid *P. aeruginosa* strains are the polar pili, whereas mucoid strains can employ both mucoid exopolysaccharide (also referred to as alginate) and pili as adhesins.

The pilus-mediated adherence of *P. aeruginosa* to epithelial cells was investigated as it is believed that nonmucoid strains are the initial colonizers in *P. aeruginosa* infections (Dogget, 1969; Hoiby, 1974). In order to elucidate the mechanism of adherence, a study was first carried out to determine the surface regions of pilin, and then to determine the part/s of pilin which is/are involved in the attachment process. The pili-mediated attachment process in other organisms has been shown to involve adhesin molecules, which are proteins organized at the tip of the pilus or along the shaft of the pilus, but are not the structural pilin proteins (Hanson and Brinton Jr., 1988; Lindberg *et al.*, 1987; Maurer and Orndorff, 1987). However, in the NMePhe pilus of *P. aeruginosa*, no other minor pilus-associated proteins, besides the structural pilin subunit, have been found. The gonoccocal pilin, which is also of the same class of NMePhe pilin, is also believed to be the adhesin in the gonoccocal attachment process (Swanson, 1973). The studies of

Schoolnik and coworkers (Schoolnik *et al.*, 1983; Rothbard *et al.*, 1984; Rothbard *et al.*, 1985) and Heckels, Saunders and their coworkers (Nicholson *et al.*, 1987) have revealed that there are two regions in the gonococcal pilin which are involved in the adherence of the gonoccocal whole cell to epithelial cells. Recently, there has been speculation that there might be other proteins associated with the gonoccocal pilus (Muir *et al.*, 1988; Parge *et al.*, 1990). The proteins have yet to be characterized and their roles have not been determined.

As a first step to elucidating the region(s) of pilin which may have an important role in the adherence of *Pseudomonas* to host cells, the surface regions of the pilin molecule were mapped using antipeptide antibodies raised against synthetic peptides corresponding to the pilin sequence of *P. aeruginosa* strain K (PAK). The PAK strain was chosen because the biological characteristics and the amino acid sequence of the pilus protein are known (Sastry *et al.*, 1983). In addition, a similar study was carried out with PAO pilin to compare the surface regions of the two pilin molecules. In this way, we could obtain crossreactive and strain-specific antipeptide antibodies. The selection of peptides to be synthesized was based on prediction algorithms from a computer program, Surfaceplot, which utilized hydrophilicity, accessibility and mobility parameters to determine the surface potential of any region from the primary sequence of the protein (Lee *et al.*, 1989; Parker *et al.*, 1986; Strynadka *et al.*, 1988).

B. Results

1. *Comparisons between predicted surface regions of PAK and PAO pilins*

The Surfaceplot prediction algorithm of Parker *et al.* (1986) suggested that there are eight potential surface regions in both PAK and PAO pilins (numbered 1-8 on Figure V.1). The heights and lengths of these regions gave indications of the degrees of their surface potential. In the case of the PAK pilin, the regions with the highest surface potential were 3, 5, 6 and 8 (residues 58-70, 89-96, 106-112 and 130-141, respectively), whereas in

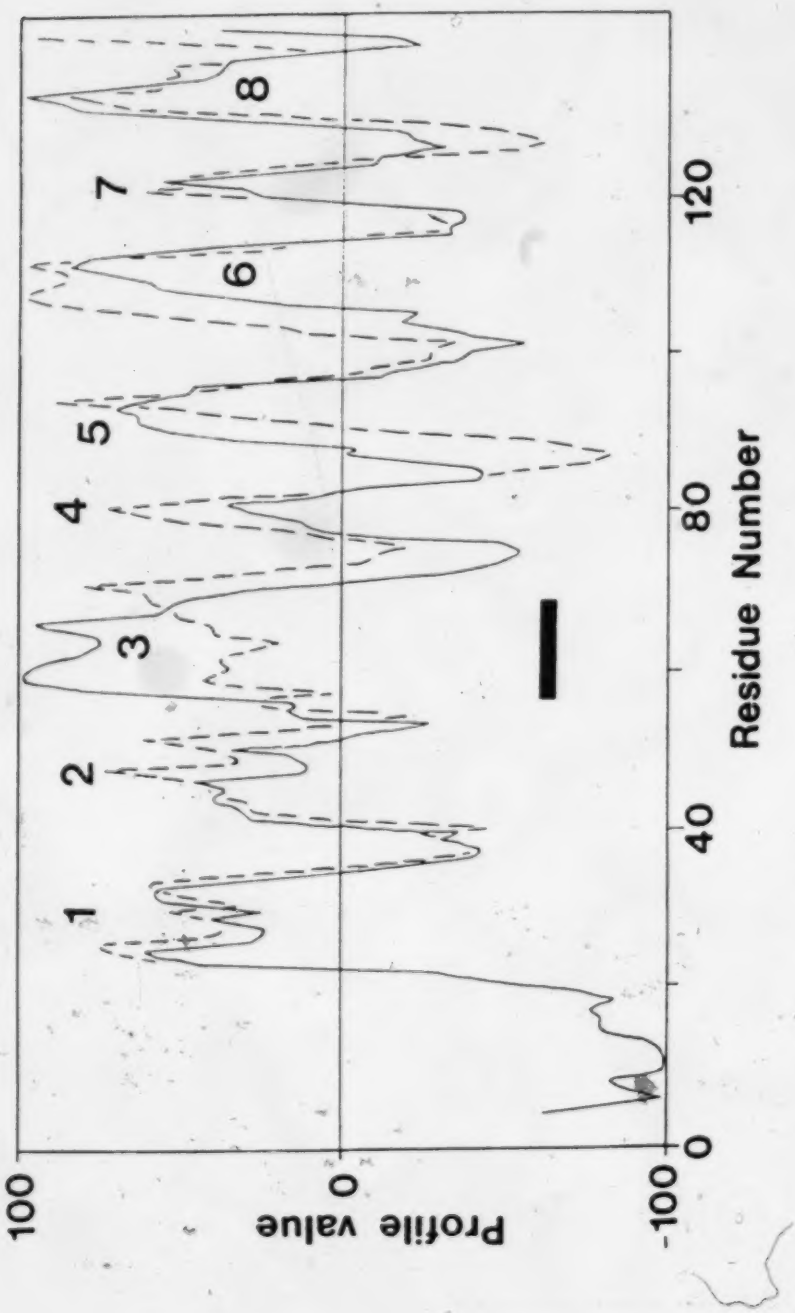


Figure V.1. Plot of the predicted surface regions of PAK (—) and PAO (----) pilins using Surfaceplot program of Parker *et al.* (1986). Profile values above zero represent predicted surface regions and those below represent hydrophobic regions. The solid bar denotes the region 58-70 where the largest difference between the surface profile values of PAK and PAO was observed.

PAO pilin, these were regions 5, 6 and 8 (residues 92-96, 105-112 and 130-140, respectively). The rest of the surface potential regions of PAO pilin were moderately high, with the exception of region 7 (residues 120-122). However, in PAK pilin, regions 2, 4 and 7 (residues 42-47, 77-81, and 120-122, respectively) all have relatively low surface potential. The main difference in the profiles was observed in the central region of the sequences of the PAK and PAO pilin molecules (represented by a solid bar in Figure V.1). The predicted surface region 3 of PAK pilin (residues 58-70) has more surface potential than the corresponding region 3 (residues 55-71) of PAO pilin, while the converse was true for region 4 (residues between 77-81). The regions of the molecule which were predicted to be buried in the interior of the molecule have large negative profile values, as depicted in Figure V.1. The N-terminal regions of both pilins are conserved and, hence, the plots overlapped with each other. It has been proposed that the highly conserved region preserves an important function of maintaining the structural integrity of the NMePhe pilin subunit-subunit interactions by being buried within the subunit (Hermodson *et al.*, 1978; Paranchych *et al.*, 1978). Synthetic peptides encompassing the predicted surface regions of both pilins were made and coupled to protein carriers [keyhole limpet hemocyanin (KLH)] to raise rabbit antipeptide antisera (see Materials and Methods Section). The amino acid sequences of the synthetic peptides are shown in Figure V.2. Two of the eight predicted surface regions have identical sequences in both pilins (regions 22-33 and 41-49). The corresponding antisera were designated anti-PAX 22-33 and anti-PAX 41-49 to indicated the sequence identity between strains. In the case of the PAK pilin, the sequence of an interior region (residues 34-40) was also synthesized to serve as a negative control.

2. *Reactivities of antipeptide antisera with PAK and PAO pili*

Direct ELISAs were performed with the microplates coated with each of the BSA-peptide conjugates to screen for the presence of its respective antipeptide antiserum (see Methods and Materials Section). Each antipeptide antiserum was subsequently assayed for

Predicted Surface Regions	Peptide Region Synthesized	Amino Acid Sequences
23-29, 31-33	PAK 22-33	P-Q-Y-Q-N-Y-V-A-R-S-E-G-NH_2
23-34	PAO 22-33	P-Q-Y-Q-N-Y-V-A-R-S-E-G-NH_2
42-47	PAK 41-49	N-P-L-K-T-T-V-E-E-NH_2
42-51	PAO 41-49	N-P-L-K-T-T-V-E-E-NH_2
58-70	PAK 58-70	K-S-G-T-G-T-E-D-A-T-K-K-E-NH_2
55-71	PAO 58-70	S-K-I-K-I-G-T-T-A-S-T-A-T-NH_2
77-78, 80-81	PAK 74-83	G-V-A-A-D-A-N-K-L-G-NH_2
78-81	PAO 75-84	G-V-E-P-D-A-N-K-L-G-NH_2
89-96	PAK 88-97	K-P-D-P-A-D-G-T-A-D-NH_2
92-96	PAO 88-97	V-A-I-E-D-S-G-A-G-D-NH_2
106-113	PAK 105-114	G-G-A-G-P-K-N-K-G-K-NH_2
105-112	PAO 105-114	G-T-S-S-P-K-N-A-T-K-NH_2
120-122	PAK 117-125	T-L-T-R-T-A-A-D-G-NH_2
120-122	PAO 117-125	T-L-N-R-T-A-D-G-V-NH_2
130-137, 141	PAK 128-144	K-C-T-S-D-Q-D-E-Q-F-I-P-K-G-C-S-K-OH
130-137, 139-140	PAO 128-144	A-C-K-S-T-Q-D-P-M-F-T-P-K-G-C-D-N-OH

Figure V.2. Amino acid sequences of the synthetic peptides which corresponded to the predicted surface regions of PAK and PAO pilins generated from Surfaceplot program. The boxed regions represented nonconserved residues between the two pilin sequences. In addition to the sequences shown, BB-Nle, was added to the N-termini of the peptides, where BB is the photoaffinity probe (benzoylbenzoic acid) used to couple the peptides to the protein carrier and Nle is the norleucine residue. PAX 22-33 and PAX 41-49 indicate sequence identity between strains in the regions of 22-33 and 41-49 of the pilin molecules.

TABLE V.1. Antipeptide antisera titers measured by direct ELISA when assayed against BSA-peptide conjugates and PAK pili.

Peptide	Corresponding Antipeptide Antiserum Titers[a]	
	BSA-peptide conjugates	PAK pili
22-33	1.2×10^{-5}	7.9×10^{-3}
34-40[c]	2.2×10^{-6}	-
41-49	2.0×10^{-5}	8.9×10^{-4}
58-70	2.0×10^{-5}	7.9×10^{-4}
74-83	2.2×10^{-5}	-
88-97	4.5×10^{-6}	9.5×10^{-3}
105-114	2.2×10^{-6}	1.1×10^{-4}
117-125	7.4×10^{-6}	5.4×10^{-3}
128-144 R1[b]	3.5×10^{-6}	1.8×10^{-5}
128-144 R2[b]	7.1×10^{-6}	8.9×10^{-6}
128-144 O1[b]	4.5×10^{-6}	7.9×10^{-6}
128-144 O2[b]	2.1×10^{-6}	8.9×10^{-6}
137-144	2.4×10^{-6}	1.0×10^{-3}

a) The antiserum endpoint titer was determined as the cutoff at an A_{405} of 0.05 U.

b) The peptides as shown in Figure V.2 were coupled to BSA and their antigenicities assayed using an anti-PAK pilus antiserum. R1 and R2 represent two different rabbit antisera to the reduced peptide. O1 and O2 represent two different antisera to the oxidized peptide (disulfide bonded between cysteine residues 129 and 142).

c) This region was used as a control. It is a predicted interior region in the protein (Parker *et al.*, 1986).

binding to its homologous pili. The results are summarized in Tables V.1 and V.2. Anti-PAK peptide antisera obtained from KLH-peptide immunogens have antibody titers that were in the range of 10^{-5} to 10^{-6}, when the binding of the antipeptide antibodies to BSA-peptide conjugates was assayed (Table V.1). Of the eight predicted surface regions for PAK pilin, only one of the antisera raised against region 3 (anti-PAK 74-83) failed to react with PAK pili in direct ELISA. A control consisting of antipeptide antibodies to PAK 34-40, a region which is not predicted to be on the surface of the protein, also failed to bind PAK pili. The antibody titers, when assayed against PAK pili, were 10- to 1000- fold less than the antibody titers assayed against their respective BSA-peptide conjugates. However, this was not the case with the anti-PAK 128-144 antisera. Of the four anti-PAK 128-144 antisera, three of these reacted as strongly with the native protein as with the BSA-peptide conjugate, with antibody titers in the range of 10^{-6}. Antipeptide antisera raised to synthetic peptides encompassing residues 22-33, 88-97, 117-125 and 137-144 (regions 1, 5, 7 and 8) gave weak responses in ELISA. Sera raised against synthetic peptides encompassing residues 41-49, 58-70 and 105-114 (regions 3, 4 and 6) gave slightly better results, with antibody titers in the range of 10^{-4}.

Region 8 of the PAO pilin (encompassing residues 128-144), like its PAK counterpart, also produced the highest antibody titer (3.2×10^{-6}) of all the anti-PAO peptide antisera which were screened, as shown in Table V.2. Most of the PAO peptides gave rise to antibody titers in the range of 10^{-5}, but two of these (anti-PAO 58-70 and 88-97) were only in the range of 10^{-4}. When all eight of the anti-PAO peptide antibodies were assayed for binding to PAO pili in direct ELISA, it was found that six of these bound to PAO pili. Even though PAO 58-70 and 88-97 did not produce antibody titers as high as some of the other PAO peptides, both of these antipeptide antisera bound to PAO pili. Anti-PAX 41-49 gave only weak binding to PAO pili. Again, as observed with the anti-PAK peptide antisera, in all instances where anti-PAO peptide antisera bound to PAO pili, the antibody titers were about 3 -1000 fold lower than the titers assayed with the corresponding BSA-

TABLE V.2. Anti-PAO peptide antisera titers measured by direct ELISA when assayed against BSA-peptide conjugates and PAO pili.

Antipeptide Antisera Against PAO Pilin Regions	End-point Titers	
	BSA-peptide Conjugates	PAO Pili
22 - 33	2.0×10^{-5}	$\sim 1 \times 10^{-3}$
41 - 49	1.6×10^{-5}	$\sim 1 \times 10^{-2}$
58 - 70	2.0×10^{-4}	6.3×10^{-4}
75 - 84	1.5×10^{-5}	1.6×10^{-4}
88 - 97	1.0×10^{-4}	1.0×10^{-3}
105 - 114	4.2×10^{-5}	-
117 - 125	4.2×10^{-5}	-
128 - 144	3.2×10^{-6}	1.8×10^{-5}

The procedures are similar to those for the PAK pili (see legend of TABLE V.1).

peptide conjugates. The antipeptide antibody titers to native pili can be expected to be lower, since many of the antibodies raised against small peptides in polyclonal sera may not recognize the corresponding peptide regions in their native conformations within the protein molecule.

3. *Reactivities of anti-pilus antisera with PAK and PAO pilin peptides*

The BSA-peptide conjugates were used to screen for linear B-cell epitopes on the pilin molecules. Anti-pilus antisera to the respective PAK and PAO pili were assayed with the synthetic peptides, and the results are summarized in Table V.3. With the PAK peptides, only the regions of residues 22-33 and 88-97 were not bound by anti-PAK pilus antiserum. PAK 74-83 was the most antigenic of the eight PAK peptides. The synthetic peptide encompassing residues 128-144 was the most antigenic of the eight PAO peptides. With the exception of PAO 88-97, the other PAO peptides displayed weaker binding to the anti-PAO pilus antiserum. In a similar manner to PAK 88-97, which did not bind to anti-PAK pilus antiserum, PAO 88-97 did not show any binding to the anti-PAO pilus antiserum.

4. *Crossreactivities of anti-PAK and anti-PAO peptide antisera with heterologous PAK and ...PAO pili in direct ELISA*

The results of the crossreactivity experiments are summarized in Table V.4. Of the six anti-PAO peptide antisera that bound to PAO pili, five of these crossreacted with PAK pili . Likewise, of the seven anti-PAK peptide antisera that bound to PAK pili, four of these crossreacted with PAO pili . The crossreactivities were observed in similar regions, i.e., the N-terminal half of the pilin molecule (residues 22-33, 41-49 and 58-70) and in the C-terminal region (residues 128-144) which contains the disulfide bond. As expected, anti-PAX 22-33 and anti-PAX 41-49 bound to both PAK and PAO pili, due to sequence homology between the two strains. Surprisingly, both the anti-PAK and anti-PAO 58-70

TABLE V.3. Antigenicities of corresponding peptide regions of PAK and PAO pilins when reacted against their respective homologous whole pilus antisera.

PAK peptide	Antigenicity[a]		PAO peptide	Antigenicity	
22 - 33	-		22 - 33	+	(4.0×10^{-4})
41 - 49	++	(1.3×10^{-5})	41 - 49	+	(2.3×10^{-4})
58 - 70	+	(4.0×10^{-5})	58 - 70	+	(5.2×10^{-4})
74 - 83	+++	(2.8×10^{-6})	75 - 84	+	(9.2×10^{-4})
88 - 97	-		88 - 97	-	(3.0×10^{-2})
105 - 114	++	(2.0×10^{-5})	105 - 114	+	(4.3×10^{-4})
117 - 125	+	(2.7×10^{-3})	117 - 125	+	(4.3×10^{-4})
128 - 144	++	(4.3×10^{-5})	128 - 144	++	(4.2×10^{-5})

a) The (-) symbol represents end-point titers of 10^{-2} or the absence of binding of peptide to antisera; the (+) symbols denote progressively higher end-point titers and stronger binding. The antigenicities of the peptides were determined using direct ELISA, where the wells of the microplate were coated with the respective BSA peptide conjugates and reacted with antisera raised against whole pili.

TABLE V.4. Crossreactivities of anti-PAK and anti-PAO antipeptide antisera with heterologous pili from PAK and PAO strains in direct ELISA.

Anti-PAK Peptide Antisera	Crossreactivities with PAO Pili	Anti-PAO Peptide Antisera	Crossreactivities with PAK Pili
22 - 33	+ (1.1×10^{-3})	22 - 33	+ (2.9×10^{-3})
41 - 49	+ (1.4×10^{-3})	41 - 49	+ (1.4×10^{-3})
58 - 70	++ (1.7×10^{-4})	58 - 70	++ (5.3×10^{-4})
74 - 83	-* (~ 10^{-2})	75 - 84	+ (2.0×10^{-3})
88 - 97	-	88 - 97	- (~10^{-2})
105 - 114	-	105 - 114	-*
117 - 125	-	117 - 125	-* (~10^{-2})
128 - 144	+++ (2.5×10^{-5})	128 - 144	++ (2.4×10^{-4})

The (-) symbol represents end-point titers of 10^{-2} or the absence of binding of antipeptide antisera; the number of (+) symbols denote progressively higher end-point titers and stronger binding; (*) these antipeptide antisera did not bind to homologous or heterologous pili.

antisera gave rise to unexpected crossreactivities, even though little sequence homology was observed in the aligned protein sequences of the two pilin proteins [only one of thirteen residues was identical (panel A, Figure V.3)]. To account for this crossreactivity, the possible sequence homology between residues 58-70 with the complete sequence of the heterologous variant protein was searched and the data is presented in Figure V.3 (panels B and C). Two tripeptide sequences of PAK 58-70 were found to be identical to regions of the PAO pilin sequence; residues 61-63 (T-G-T) and 66-68 (A-T-K) of PAK pilin share homology with residues 104-106 and 112-114 of PAO pilin (panel B, Figure V.3). These two regions are in close proximity to each other and could account for the ability of anti-PAK 58-70 to crossreact with PAO pili. The PAK 58-70 region also shared some homology with residues 61-73 and 71-84 of the PAO pilin. These homologies involved five identical residues out of a stretch of thirteen residues. The homology between the PAK 58-70 and PAO 61-73 regions was obtained by shifting the sequence by only 3 residues. The other two possible homologies (between PAK 58-70 and PAO 71-84, and between PAK 58-70 and PAO 101-116) involved insertions of amino acid spaces to achieve maximum sequence homology. The latter would suggest that crossreactivities result from critical residues being brought into close contact with each other via folding of the polypeptide backbone to give rise to a discontinuous epitope.

A homology search of the PAO 58-70 region with the complete PAK pilin sequence was also carried out. The PAO 58-70 region showed sequence homology with PAK 55-67 following a shift of 3 residues [five identical residues out of thirteen (panel C, Figure V.3)]. This homology is the same as that observed between the PAK 58-70 with the PAO 61-73 regions (Panel B, Figure V.3). A second region of homology was observed between the PAO 58-70 and the PAK 10-21 regions. Maximum sequence homology was observed between PAO 58-70 and PAK 111-123 (six identical residues out of thirteen, panel C, Figure V.3).

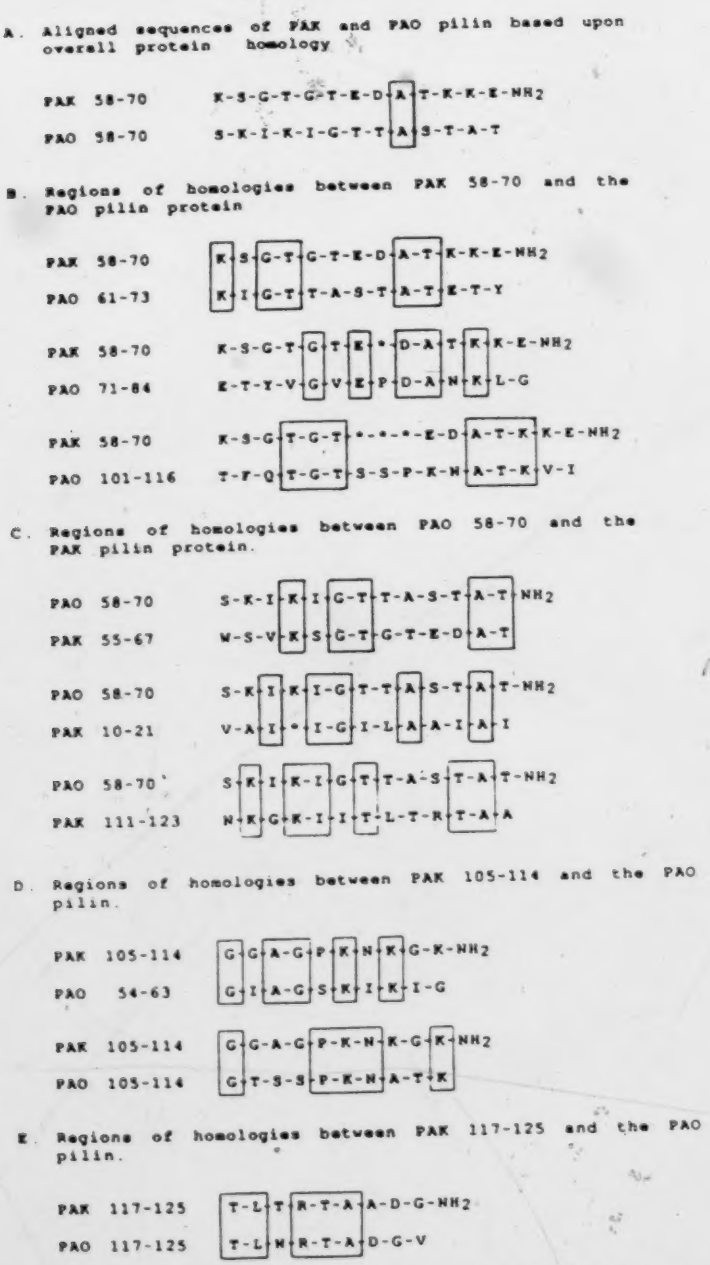

A. Aligned sequences of PAK and PAO pilin based upon overall protein homology
PAK 58-70 K-S-G-T-G-T-E-D-A-T-K-K-E-NH2
PAO 58-70 S-K-I-K-I-G-T-T-A-S-T-A-T
B. Regions of homologies between PAK 58-70 and the PAO pilin protein
PAK 58-70 K-S-G-T-G-T-E-D-A-T-K-K-E-NH2
PAO 61-73 K-I-G-T-T-A-S-T-A-T-E-T-Y
PAK 58-70 K-S-G-T-G-T-E-*-D-A-T-K-K-E-NH2
PAO 71-84 E-T-Y-V-G-V-E-P-D-A-N-K-L-G
PAK 58-70 K-S-G-T-G-T-*-*-*-E-D-A-T-K-K-E-NH2
PAO 101-116 T-F-Q-T-G-T-S-S-P-K-N-A-T-K-V-I
C. Regions of homologies between PAO 58-70 and the PAK pilin protein.
PAO 58-70 S-K-I-K-I-G-T-T-A-S-T-A-T-NH2
PAK 55-67 W-S-V-K-S-G-T-G-T-E-D-A-T
PAO 58-70 S-K-I-K-I-G-T-T-A-S-T-A-T-NH2
PAK 10-21 V-A-I-*-I-G-I-L-A-A-I-A-I
PAO 58-70 S-K-I-K-I-G-T-T-A-S-T-A-T-NH2
PAK 111-123 N-K-G-K-I-I-T-L-T-R-T-A-A
D. Regions of homologies between PAK 105-114 and the PAO pilin.
PAK 105-114 G-G-A-G-P-K-N-K-G-K-NH2
PAO 54-63 G-I-A-G-S-K-I-K-I-G
PAK 105-114 G-G-A-G-P-K-N-K-G-K-NH2
PAO 105-114 G-T-S-S-P-K-N-A-T-K
E. Regions of homologies between PAK 117-125 and the PAO pilin.
PAK 117-125 T-L-T-R-T-A-A-D-G-NH2
PAO 117-125 T-L-M-R-T-A-D-G-V

Figure V.3. Can sequence homologies explain crossreactivities? Sequence homology to residues 58-70 of PAK was examined in other regions of the PAK protein (internal homologies) and for homology in the protein sequence of the heterologous PAO pilin (external homologies). A similar search was carried out with PAO 58-70, PAK 105-114 and PAK 117-125. The search was carried out using a commercially available program, Sequence Search (Synthetic Peptide Inc., University of Alberta, Edmonton, Canada). The program searches for short peptide homologies ranging from 25-100% to percent to any selected proteins. * represent insertions of residues to maximize homology.

In addition, anti-PAO 75-84 was also able to bind to the PAK pili. However, anti-PAK 75-84 did not bind to PAK pili, nor was it able to crossreact to PAO pili. The surface profile plot (Figure V.1) showed that this region of the PAO pilin has a much higher surface potential than the corresponding PAK pilin. The two amino acid substitutions of Ala-Ala (PAK 76-77) to Glu-Pro in PAO seemed to have a pronounced effect on the surface accessibility of this region. The Glu substitution has the effect of increasing the hydrophilicity and the Pro substitution might enhance the accessibility of this region. Obviously, sequence homology cannot guarantee crossreactivity. Anti-PAK 105-114 and anti-PAK 117-125 failed to crossreact with PAO pili (Table V.4), even though these regions have 50-55% identity (Panels D and E, Figure V.3).

Regions that gave rise to strain-specific antibodies were PAK 88-97, 105-144 and 117-125 and PAO 88-97 (Table V.4). The region of residues 88-97 was expected to give rise to strain-specific antibodies, due to the lack of sequence homology between two pilins (2 residues of identity out of 10, Figure V.2). A search for maximum homology of the region encompassing residues 88-97 with heterologous pilin sequences only showed homology for a short tripeptide sequence (an A-D-G sequence in PAK 92-94 and PAO 122-124 and a G-A-G sequence in PAO 94-96 and PAK 106-108). It seems reasonable that a minimum number of identical residues is required for crossreactivity; and 2 or 3 residues of identity are not sufficient to create crossreactivity. The results suggested that a simple comparison of aligned protein sequences of different pilin strains would not necessarily give an indication for the production of crossreactive antibodies or strain-specific antibodies. A careful search of the region of interest with the sequence of the whole crossreacting protein is necessary to predict potential crossreactivity.

C. Discussion

In order to evaluate the possible similarities and differences in the surface regions of PAK and PAO pili, predicted surface profiles were first examined as shown in Figure V.1. The prediction algorithms were based on hydrophilicity, accessibility and mobility parameters. The use of a combination of three parameters instead of a single parameter has been shown to produce better predictions of surface antigenic sites (Lee *et al.*, 1989; Parker *et al.*, 1986; Strynadka *et al.*, 1988). The PAK and PAO pilin molecules share approximately 49% homology between their amino acid sequences (Sastry *et al.*, 1985). The composite surface profile plots of both PAK and PAO pilins, revealed very similar profiles, and eight potential surface regions were predicted. In fact, analyses of a number of pilins from *P. aeruginosa* strains (CD4, P1 and K122-4) have also predicted eight overlapping potential surface regions (Pasloske *et al.*, 1988), with regions 3, 5, 6 and 8 showing the highest predicted surface potentials. Secondary structure predictions, reported by Paranchych and coworkers (Paranchych, 1989; Sastry *et al.*, 1985), did not reveal significant differences between the PAK and PAO pilins. Watts *et al.* (1983) reported that PAO and PAK pilins have identical circular dichroic spectra with 47% α-helical content. The pilus is composed of pilin molecules joined together through subunit-subunit interactions in a helical fashion (Folkhard *et al.*, 1981; Watts *et al.*, 1983a). Thus, not all the predicted surface regions on the pilin subunit would be expected to be exposed after assembly of the pilin subunits in the pilus.

Peptides corresponding to the *Pseudomonas* pilin sequence were synthesized, covering 56% (81 of 144 residues) of the whole pilin molecule. Of the regions with highest surface potential regions 3, 5, 6 and 8 for PAK pilin, and regions 5, 6 and 8 for PAO pilin, antipeptide antibodies directed against region 8 (residues 128-144) had the highest antibody titers in both instances. The region of residues 128-144 of both PAK and PAO pilins, have two cysteine residues which form a disulfide loop in the native molecule. The presence of a disulfide bond in the peptide could have given rise to a more stable

conformation which might resemble the native structure in the protein. This stabilization could result in the generation of antibodies with a higher affinity for the protein molecule. Examples of the use of such looped peptides to produce antipeptide antibodies to lyzozyme, hepatitis-B surface antigen and human chorionic gonadotropin have been demonstrated (Arnon *et al.*, 1971; Dressman *et al.*, 1984; Stevens *et al.*, 1986). Interestingly, immunization with the reduced form of this peptide also gave rise to good antibody titers, as shown by the PAK pilin study. However, it was possible that the peptide may have reoxidized during immunization, since its free sulfhydryl groups were not alkylated. An antiserum raised against an analog of this peptide with the cysteine-129 substituted by an alanine residue bound weakly to PAK pili in direct ELISA even though high antibody titers were obtained (Table IV.1, Chapter IV). This suggested that the disulfide bridge might be important in raising antipeptide antibodies that bind to the native protein in this region (see Chapter IV). Antibodies raised against a short synthetic peptide, corresponding to the last eight residues of the C-terminal end of the PAK pilin (residues 137-144), also gave rise to high antibody titers, but bound poorly to the PAK pili. The linear peptide sequence can be very immunogenic but these antibodies may not be able to recognize the corresponding region in the protein molecule. The exposure of this region on pili seems to be important in the adhesion of *P. aeruginosa* to epithelial cells (see Chapter VI).

Anti-PAK 128-144 have been shown to crossreact with PAO pili (see Chapter IV). In this study, it was shown that anti-PAO 128-144 was also able to crossreact very well with PAK pili. This region of pilin is semi-conserved (nine of seventeen amino acids) and the disulfide bridge may induce a structural configuration to enable good recognition by heterologous antipeptide antibodies. Alternatively, the antipeptide antibodies were simply recognizing some of the conserved amino acid residues. Due to some sequence homology, it is not unusual to observe crossreactivity when an antiserum raised against a synthetic peptide is assayed against another variant of the same protein. Recently, it was demonstrated that antibodies raised against a peptide corresponding to a region of the A-

subunit of pertussis toxin was able to bind to the A-subunit of cholera toxin because of some sequence homology (seven of twelve amino acid residues) between the two proteins (Burns *et al.*, 1987). Detection of crossreactivity between tetanus and botulinum toxins by an antipeptide antibody has also been reported (Halpern *et al.*, 1989).

The binding of whole pilus PAK and PAO antiserum to the synthetic peptides was assayed using direct ELISA with the BSA-peptide conjugates (Table V.3). Anti-PAO pilus antiserum bound to seven of eight synthetic peptides, while anti-PAK pilus antiserum bound to six of eight synthetic peptides. The region of residues 88-97 failed to bind either antisera. Interestingly, it is to this same region that antipeptide antibodies provided strain specificity. Although antibodies raised against the whole protein generally recognize conformational epitopes, these results clearly show that the linear sequences in the native pilin molecule are immunogenic. These results are in contrast with those of Lerner and coworkers (Green *et al.*, 1982) who found that antibodies to the intact influenza virus hemagglutinin did not react with any of 20 synthetic peptides corresponding to amino acid sequences of hemagglutinin. Yet, antipeptide antibodies to 18 of these 20 peptides were able to react with hemagglutinin. As suggested by Lerner (1982), peptide immunization can generate antibody specificity (both crossreactive and strain-specific) that cannot be obtained in other ways.

The major antigenic determinant of the PAK pilin has been reported to be a conformational epitope situated between the region encompassing 82-110 (Watts *et al.*, 1983). This region of the molecule is also believed to be the T-cell recognition site (Smart *et al.*, 1988). Isolated T-cell clones that bind to PAK pili failed to recognize the synthetic PAK 88-97 peptide (B. Singh, K.K. Lee and R.S. Hodges, unpublished data). This suggested that the major immunogenic determinant consists of more than these 11 amino acid residues, and that a longer peptide is needed for binding. Two other peptides, PAK 100-110 and 92-110, which were synthesized also failed to be recognized by these T-cell clones (B. Singh, K.K. Lee and R.S. Hodges, unpublished data). The anti-PAK pilus

antiserum bound very well to the PAK 137-144 peptide but the anti-PAK 137-144 antipeptide antiserum bound poorly to the PAK pili. In the case of the anti-PAK 74-83 antipeptide antiserum, the binding was hardly detectable (better binding could be obtained if the antiserum was first concentrated). These two peptides could have assumed conformations which were quite different from the conformation on the pilin molecule. This emphasizes the importance of the method of peptide presentation to the immune system. The peptide must be presented in a manner to mimic its conformation in the native protein. Failure of antipeptide antiserum to recognize the native protein cannot be interpreted that the region is not surface exposed. The combination of antipeptide antisera and the anti-PAK pilus antiserum results verified the success of Surfaceplot in that all regions predicted to be on the surface were found to be exposed on native pili.

In conclusion, the results of these studies have pointed to the complexity of the immune system and the difficulty of predicting regions of a protein molecule which could give rise to strain-specific and crossreactive antibodies. An explanation for crossreactivity or specificity in the presence of reasonable sequence homology remains an important area of investigation to be solved in molecular immunology. These results also suggested the possibility of raising strain-specific antipeptide antisera which could be useful in diagnosing the *P. aeruginosa* strains involved in an infection, and of raising crossreactive antisera which may be useful in synthetic vaccine development. We have also demonstrated the importance of the disulfide-bridged region of PAK pilin in the pilus-mediated adherence of *P. aeruginosa* to BECs (Chapter VI). The 128-144 peptide, encompassing the C-terminal disulfide loop, is highly immunogenic when coupled to a carrier protein, and should present itself as a good candidate for raising crossreactive antiserum to block *Pseudomonas* adherence.

Chapter VI. Investigation of the Cell-Surface Binding Domain of *P. aeruginosa* PAK Pilus

A. Introduction

The NMePhe pilus of *P. aeruginosa* consists of a polymer of a thousand or more single subunits arranged in a helical fashion. There has been no firm evidence of other proteins associated with the pilus. This is very different from type 1, Pap and S pili, where other proteins have been shown to be associated with the pilus (Abraham *et al.*, 1987; Hanson and Brinton, 1988; Lindberg *et al.*, 1987; Maurer and Orndoff, 1987; Schmoll *et al.*, 1989). The adhesin, a molecule responsible for conferring the binding ability to the pilus, is one of these minor proteins. In the Pap pilus, the papG molecule is the adhesin and it is associated with at least two other proteins, papE and papF, at the tip of the pilus (Lund *et al.*, 1988). In the absence of other minor proteins associated with the NMePhe pilus of *P. aeruginosa*, it might be possible to delineate a region of the pilin molecule which confers the adhesion property to the pilus.

In order to elucidate the cell-binding domain of *P. aeruginosa* pilus, antipeptide antibodies were raised to peptides corresponding to predicted surface regions of PAK pilin. The antipeptide antibodies that bind to a particular sequence that contains part of the binding domain or is located near the binding domain should block the pilus from adhering to cell surface receptors either directly or indirectly (sterically). In addition, the synthetic peptides were used to probe directly for the receptor-binding domain of the pilus. One previous piece of evidence that suggested that the C-terminal portion of the PAK pilin was important for binding to cell-surface receptors came from the studies of Paranchych *et al.* (1985). They showed that a PAK fragment, corresponding to residues 121-144, inhibited the binding of PAK pili to BECs. This finding has now been extended with studies employing synthetic peptides which correspond to the C-terminal region of the PAK pilin.

B. Results

1. *The effects of polyclonal antipeptide antibodies on PAK pili binding to human buccal ...epithelial cells*

The antibodies raised against peptides corresponding to different regions of the PAK pilin were assayed for their abilities to bind to PAK pili, and also to examine their effects on pili binding to BECs. The results are summarized in Table VI.1. When Fab fragments generated from antipeptide antibodies were incubated with pili prior to their addition to BECs, only antibodies raised against the C-terminal disulfide loop region were able to inhibit the adherence of PAK pili to BECs (Table VI.1 and Figure VI.1). All four antisera raised against PAK 128-144 peptides (reduced and oxidized) reduced the binding of PAK pili to BEC by 36 - 72% of that of the control (which consisted of pre-immune Fab fragments from one of the rabbits used for immunization). A murine monoclonal antibody, PK99H, directed against the C-terminal region of PAK pilin which served as a positive control, was also effective in reducing the adherence of PAK pili to BECs to 64% of the control. In addition, an antipeptide antiserum raised against a shortened peptide of the C-terminal end (137-144) also provided about 64% inhibition of pili binding to BECs. Anti-PAK 117-125 failed to inhibit binding even though this region is adjacent to the disulfide bridge. Interestingly, an antiserum raised against an analog of the PAK 128-144 peptide, where cysteine-129 was replaced by an alanine residue, was also able to inhibit PAK pili binding by 47% of the control. These results are more easily visualized in Figure VI.1. Antipeptide antibodies raised to regions at the N-terminal and the hypervariable central portion of PAK pilin failed to inhibit pili binding to these BECs. Only those antibodies directed against the C-terminal disulfide-bridged region were able to block the adhesion of pili to cell surface receptors.

Peptides to which Fab fragments were generated	% PAK pili Binding to BECs
22-33	100.0 ± 2.5
34-40	82.1 ± 20.3
41-49	94.8 ± 8.9
58-70	97.3 ± 4.6
74-83	100.5 ± 8.5
88-97	101.5 ± 8.7
105-114	93.4 ± 18.9
117-125	99.5 ± 1.5
128-144 R1	72.0 ± 4.9
128-144 R2	62.3 ± 0.1
128-144 O1	70.6 ± 2.5
128-144 O2	35.7 ± 7.6
137-144	36.4 ± 3.0
128/A-129*	53.3 ± 0.5
PK99H	64.6 ± 2.5
Preimmune	100.0 ± 13.9
Control	92.7 ± 11.3

TABLE VI.1. The effect of antipeptide Fab fragments on the binding of PAK pili to human buccal epithelial cells (BECs). The preimmune Fab obtained from one of the rabbits used for immunization served as a negative control. PK99H is an anti-PAK pilin monoclonal antibody. All Fabs were diluted such that their final titer as measured by ELISA to PAK pili was 10^3. The data represents the average of three independent sets of experiments. * 128/A-129 is the peptide 128-144 with cysteine-129 replaced by an alanine residue.

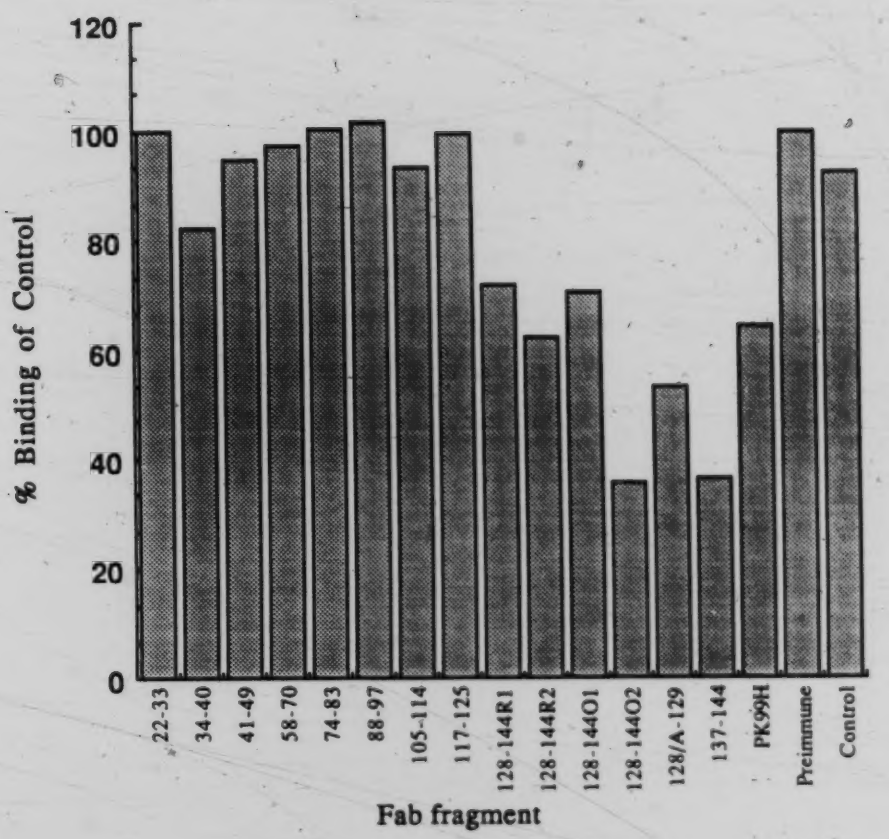


Figure VI.1. Inhibition of PAK pili binding to human buccal epithelial cells (BECs) by Fab fragments generated from antipeptide antibodies. The binding of PAK pili is represented as a percentage of the control, in which no Fab fragments were present in the assay.

2. *The effects of murine monoclonal antibodies raised against PAK pili on pili binding to ...human buccal epithelial cells*

Four monoclonal antibodies raised against PAK pili (PK3B, PK34C, PK41C and PK99H), determined to be of the immunoglobulin G class, have been characterized for these studies (Doig *et al.*, 1990). The apparent association constants of these MAbs were determined by competitive ELISA and are summarized in Table VI.2. The assays were carried out with whole pili, fragments of the PAK pilin, or with synthetic peptides. PK41C did not bind to any of the PAK pilin fragments generated by chymotryptic digestion. It bound to PAK pili and crossreacted with the PAO pili. PK3B also failed to bind to any of these fragments. It specifically bound PAK pili but did not bind PAO pili. PK99H failed to bind to the PAK 31-53 and PAK 54-120 fragments. However, PK99H had a high affinity for the 121-144 chymotryptic fragment. This 121-144 fragment contained the disulfide bridge. Interestingly, PK99H was able to bind both the reduced and oxidized synthetic PAK 128-144 peptides. Furthermore, PK99H also bound to a short linear sequence at the extreme end of the C-terminal (residues 137-144). The peptide sequence prior to the disulfide bridge, residues 117-125, failed to interact strongly enough to allow the measurement of its binding affinity to PK99H. PK99H showed specificity for PAK pili only. PK34C bound strongly to the 121-144 fragment with an apparent association constant of $5.9 \times 10^7\ M^{-1}$. In contrast to PK99H, the short peptide of residues 137-144 was not bound by PK34C. PK34C only interacted with the PAK 128-144 peptide when it was oxidized suggesting the importance of the disulfide loop in forming the epitope for this particular antibody. In addition to the disulfide loop, part of the epitope of PK34C consists of some of the residues located prior to the disulfide bridge because the PAK 117-125 peptide also had an equally high affinity for the antibody, as evident by the apparent association constant. Another dissimilarity between PK34C and PK99H was that PK34C bound both PAK and PAO pili.

TABLE VI.2. Apparent association constants of PAK pili monoclonal antibodies to the PAK pilin fragments and to PAK and PAO pili determined from competitive ELISA data as described by Nieto *et al.* (1984).

Fragment PAK Pilin	PK99H	PK34C	PK3B	PK41C
1 - 30*	ND	ND	ND	ND
31 - 53*	NE	NE	NE	NE
54 - 120*	NE	NE	NE	NE
121 - 144*	9.9×10^6 M^{-1}	5.9×10^7 M^{-1}	NE	NE
117 - 125	NE	7.5×10^5 M^{-1}	ND	ND
128 - 144 (reduced)	1.0×10^8 M^{-1}	NE	ND	ND
128 - 144 (oxidized)	3.4×10^7 M^{-1}	7.6×10^5 M^{-1}	ND	ND
137-144	2.4×10^6 M^{-1}	NE	ND	ND
PAK pili	300 ml/g[a]	380 ml/g	100 ml/g	1000 ml/g
PAO pili	NE	5.6 ml/g	NE	6.1 ml/g

* Chymotryptic fragments of PAK pilin.

a - affinity constants for MAb binding to purified pili are expressed in ml/g rather than as molar affinity constant because of the polydispersed size distribution of purified pili.

ND - not done

NE - no effect

The effects of the four monoclonal antibodies on the binding of *P. aeruginosa* to BECs were assayed. Both the PK41C and PK3B had no effect on the binding of PAK pili to BECs. The epitope of the PK41C appears to be located in the N-terminal of the PAK pilin, since both the PAK 1-30 fragment and the synthetic PAK 22-33 peptide were bound by this MAb (Doig *et al.*, 1990). PK3B failed to react with any of the PAK pilin fragments or synthetic peptides. The epitope may be a conformational epitope consisting of various parts of the pilin molecule brought together when the protein is folded (Doig *et al.* 1990).

Unlike PK3B and PK41C, both PK99H and PK34C were able to inhibit bacterial whole cell binding to BECs, and the data is presented in Table VI.3. In the data shown in Table VI.3, a solution of the Fab fragment of 100 mg/ml concentration and a ratio of bacteria to BECs between 1000:1 to 1500:1 were chosen, such that a significant level of inhibition could be assessed with a minimal amount of Fab needed in the assay mixture. Hence, a maximal inhibition of binding would not be expected (the percent inhibition is given in the parenthesis in Table VI.3). PK99H was only effective in blocking strain PAK, and to a small extent strain 492c. PK34C inhibited all pilus-producing *P. aeruginosa* strains to varying degrees. Normal mouse IgG had no effect on *P. aeruginosa* binding to BECs.

3. *The ability of AcPAK 128-144 to mimic the PAK binding to cell surface receptors*

The studies of Doig *et al.* (1988) have shown that the PAK whole cells bound to a single class of receptors on BECs and TECs (tracheal epithelial cells). These workers have also shown that purified PAK pili bound to BECs in a concentration-dependent manner. In addition, both PAK pili and anti-PAK pilus Fab fragments inhibited bacterial whole cell binding to BECs. A competitive inhibition pattern of the bacterial whole cell binding to BECs by PAK pili was indicative that both, bacterium and pili, bound to the same receptors on the cell surfaces. To delineate the region of PAK pilin responsible for this receptor-binding domain, a number of fragments of the PAK pilin and synthetic peptides were

TABLE VI.3. Effect of Fab fragment of MAbs PK99H and PK34C on the binding of *P. aeruginosa* to BECs

Strain	Bacteria bound/BEC[a]		
	Control[a]	PK99H	PK34C
PAK	35.2 ± 1.4	25.6 (72.7) ± 1.0[c]	23.8 (67.5) ± 1.7[c]
PAO	50.5 ± 2.0	55.5 (110) ± 11.1	45.9 (91) ± 0.7[c]
HD1	38.0 ±3.6	31.0 (81.6) ± 3.5	31.3 (82.5) ± 1.1[c]
492c	30.9 ± 0.2	23.8 (77.1) ± 0.5[c]	26.2 (84.8) ± 1.4[c]
P1	36.3 ± 2.5	34.7 (95.6) ± 5.9	29.5 (81.3) ± 0.2[c]
K122-4	41.8 ± 1.5	38.3 (91.8) ± 2.8	28.0 (67.1) ± 0.4[c]
PAK/3	13.1 ± 1.4	12.0 (91.9) ± 0.7	12.2 (93.3) ± 0.6

[a] The concentration of PK99H and PK34C Fab used in the inhibition assays was 100 μg/ml and had a titer of 10^3 by ELISA, using PAK pili as the antigen (coated at 1 μg per well). Given is the mean ± the standard deviation. The percent of control is given in parentheses.

[b] Control value when 100 μg of Fab fragments per ml produced from normal mouse IgG was added. No difference was noted between these values and those from tubes to which no Fab fragments were added.

[c] The significant difference ($P < 0.05$) was determined by using the Student *t* test.

TABLE VI. 4. The effect of natural and synthetic peptide fragments of PAK pilin on the adherence of *Pseudomonas aeruginosa* PAK to BECs

Fragment	Concentration (μM)	Adhesion index % of Control
PAK 121-144[a]	25	379[b]
PAK 54-120[a]	25	102
PAK 31-53[a]	25	110
PAK 134-144	25	104
PAK 116-144 reduced[c]	15[d]	287[e]
PAK 116-144 oxidized[c]	15[d]	1060[e]

a - chymotryptic fragments

b - determined at a ratio of 3061:1 (bacteria:BECs) and as direct competition assay

c - N^{α}-acetylated synthetic peptides

d - the concentration of the peptides was normalized to an OD_{280} of 0.183 (about 15 μM)

e - determined at a ratio of 2240:1 (bacteria:BECs) and as a direct competition assay.

assessed for their abilities to inhibit bacterial whole cell binding to BECs. The results of these experiments are summarized in Table VI.4. The results are represented as a percentage of the control or adhesion index. The two PAK pilin fragments, residues 31-53 and 54-120, did not inhibit PAK binding to the epithelial cells. However, the fragment of residues 121-144 enhanced the binding by more than three fold. Synthetic peptides were used to extend further this result. The reduced PAK 116-144 peptide was also able to enhance PAK whole cell binding, as indicated by an approximate 3-fold increase in the adhesion index. However, the biggest increment in the enhancement of PAK whole cell binding to BECs (10-fold) was obtained when the disulfide bridge was restored to the PAK 116-144 peptide. The short PAK 137-144 peptide had no effect on the binding of bacteria to BECs.

Synthetic peptides, long enough to just cover the disulfide loop, were used to investigate the binding of the peptides to cell-surface receptors. Both the reduced and oxidized AcPAK 128-144 peptides were shown to bind to BECs in a concentration-dependent manner (Figure VI.2). Saturation of binding to BECs by the oxidized peptides was reached at about 30 μM, but the reduced conformer did not reach a saturation point. The concentration at which the reduced and oxidized AcPAK 128-144 peptide occupy 50% of the receptor sites (apparent dissociation constant) was approximately 9.9 μM and 6.40 μM, respectively.

Inhibition assays were carried out with the synthetic AcPAK 128-144 peptides to assess their abilities to block PAK pili binding to BECs. Both the reduced and oxidized peptides were able to inhibit pili binding in preliminary studies. In subsequent experiments, the inhibition of PAK pili binding to BECs was assayed with a fixed concentration of the reduced AcPAK 128-144 peptides, together with varying concentrations of PAK pili. For each set of data points, different, but fixed concentrations of the reduced peptides were used. The nature of this inhibition was investigated using the reduced peptide because of the limited availability of the oxidized conformer. The binding

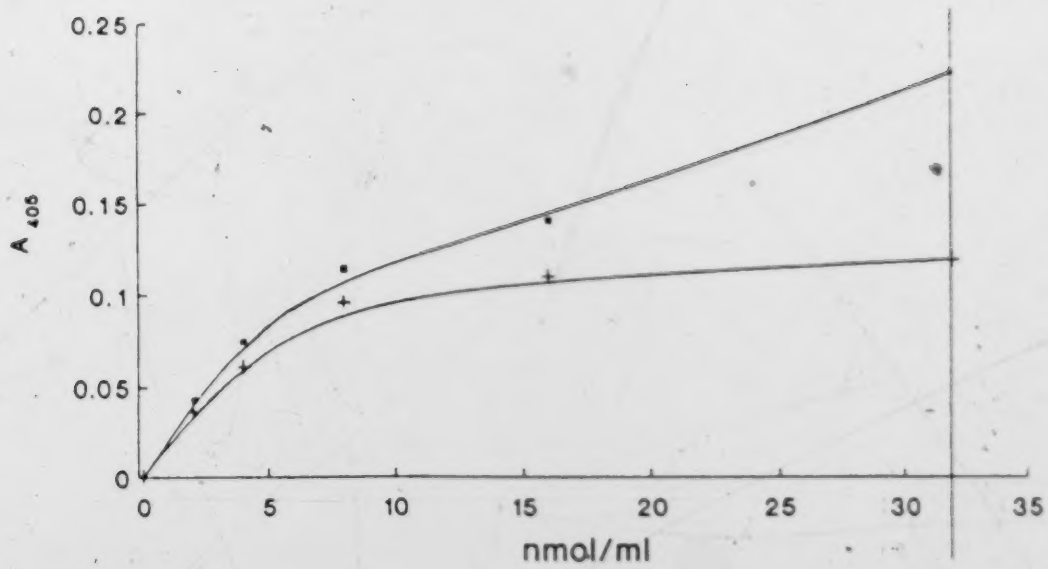


Figure VI.2. Binding of reduced (■) and oxidized (+) AcPAK 128-144 peptide to human BECs. Binding of the synthetic peptides to BECs was determined by a whole cell ELISA utilizing the monoclonal antibody, PK99H (which binds to reduced and oxidized AcPAK 128-144), to quantitate the amount of synthetic peptide bound to the surface of BECs.

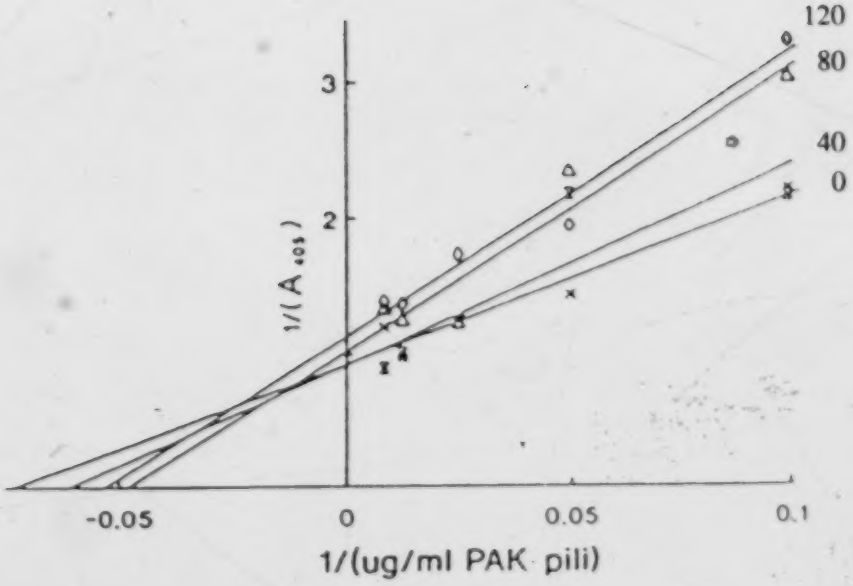


Figure VI.3. Modified Lineweaver-Burk plot of the binding of PAK pili to human BECs in the presence of 0 (▫). 40 (x), 80 (△), and 120 (◇) nmole of peptide AcPAK 128-144 per ml.

assays were analyzed using modified Lineweaver-Burk plots, as shown in Figure VI.3. The variation of the maximum absorbance at 405 nm was relatively constant unlike the changes in the apparent association constants. This pattern of inhibition was similar to that of a competitive inhibition pattern.

4. *Binding of alkaline phosphatase-peptide conjugate to BECs*

The successes of the synthetic peptide binding to cell surface receptors have led to the development of a more simple approach to assay this binding process. The synthetic peptide, PAK 121-144, was made with the benzoylbenzoyl photoreactive crosslinker and conjugated to a reporter molecule, alkaline phosphatase (AP). The binding of the AP-24mer to BECs is shown in Figure VI.4. The readings have been normalized to 10^5 BECs. The AP-24mer conjugate showed a concentration-dependent binding to the cells, and approached saturation at a concentration of about 0.6 μM. Because of the limited availability of the conjugates, higher concentrations were not used. When the data was analyzed by transformation of the data into a double reciprocal plot, a straight line was obtained (Figure VI.5), suggesting a single binding site. From this data, an apparent dissociation constant of 0.94 μM was obtained.

Interestingly, PAK pili, which had been coated onto the wells in the ELISA plates, were also bound by the AP-24mer conjugates. The binding data is shown in Figure VI.6. The AP-17mer was also able to bind to PAK pili but with a much lower A_{405} signal (data not shown). A control, using just the alkaline phosphatase alone, did not bind to the PAK pili. A double reciprocal analysis of the AP-24mer binding data (Figure VI.7) showed that the AP-24mer conjugate had an apparent association constant of 0.24 μM. This ability of the synthetic peptides to interact with the pilus appears to mimic a kind of pilin-pilin interaction which may be involved in the assembly of homopolymeric pili. Such pilin-pilin interactions were also observed by Abraham and Beachey (1987), who found that a 13-

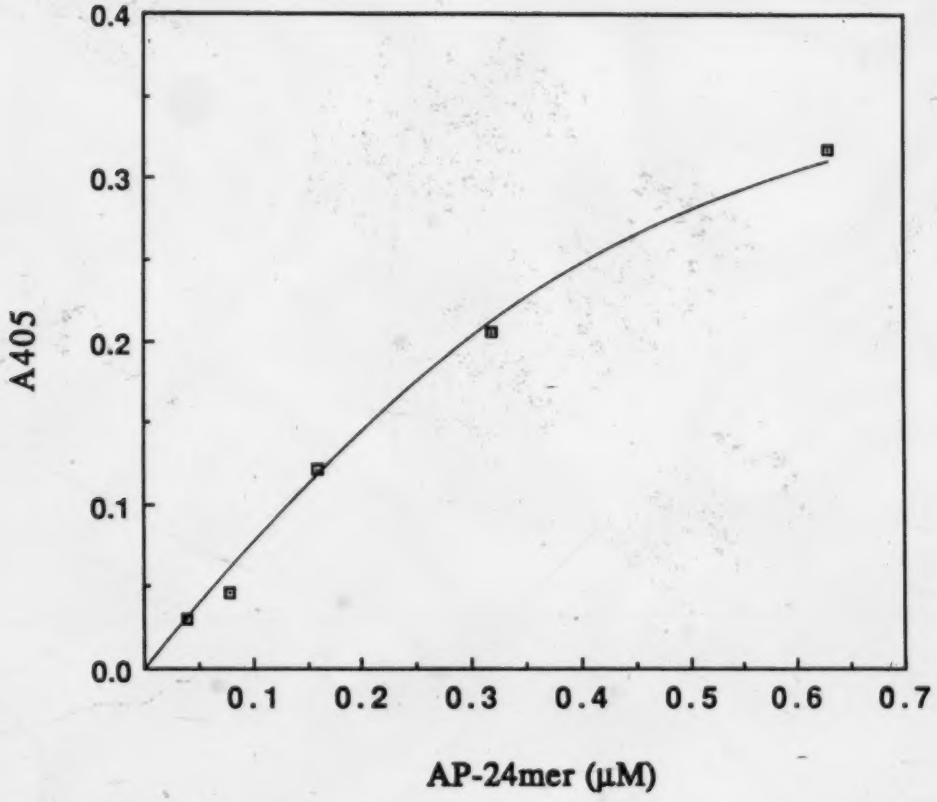


Figure VI.4. Binding of PAK 121-144 - alkaline phosphatase (AP-24mer) conjugate to BECs. These assays were carried out using an approach similar to whole cell ELISA, where varying concentrations of AP-24mer and the BECs were incubated at 37 °C. The A_{405} readings have been normalized to 10^5 BECs.

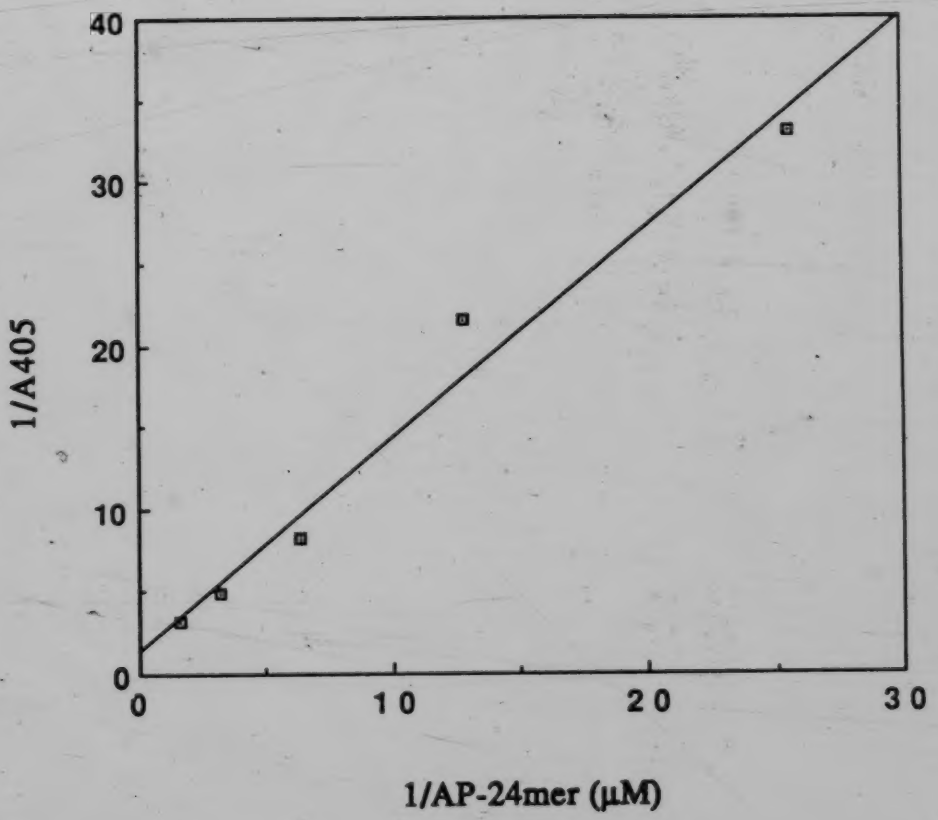


Figure VI.5. Double reciprocal plot of the binding data in Figure VI.4. The plot shows a straight line with a linear regression coefficient of 0.97.

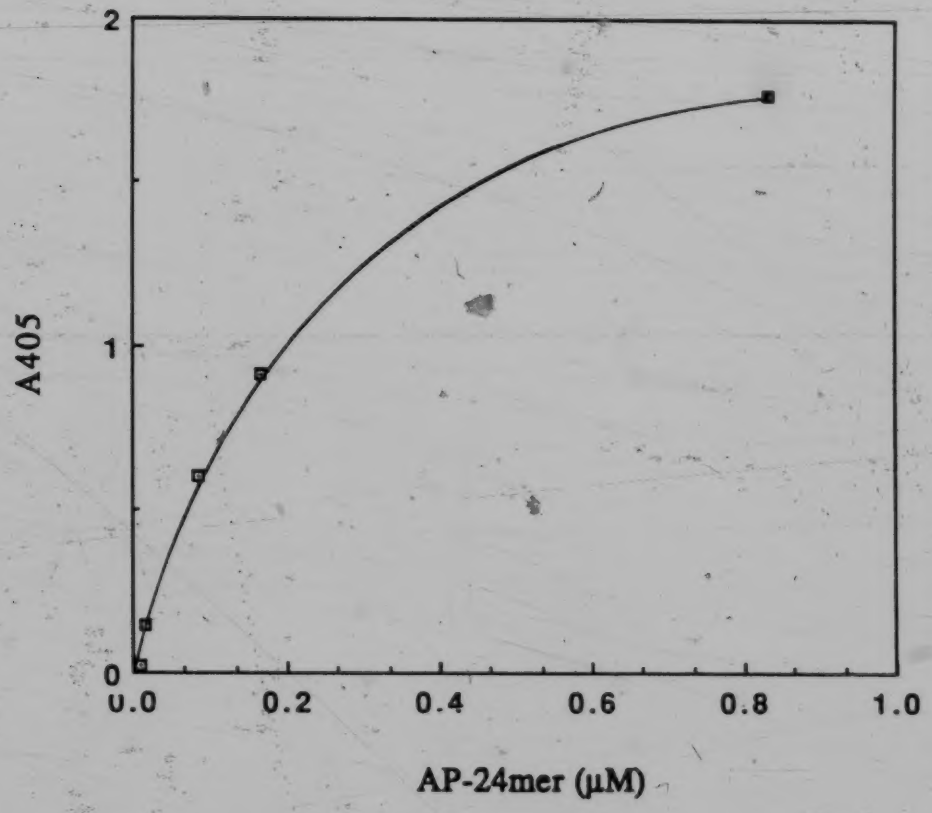


Figure VI.6. Binding of AP-24mer conjugate to PAK pili. The PAK pili were coated onto the wells in an ELISA plate and the conjugates were incubated at 37 °C for an hour with gentle agitation. Following several washes, 100 ul of p-nitrophenylphosphate (1mg/ml), were incubated for 30 min and the A_{405} readings were taken.

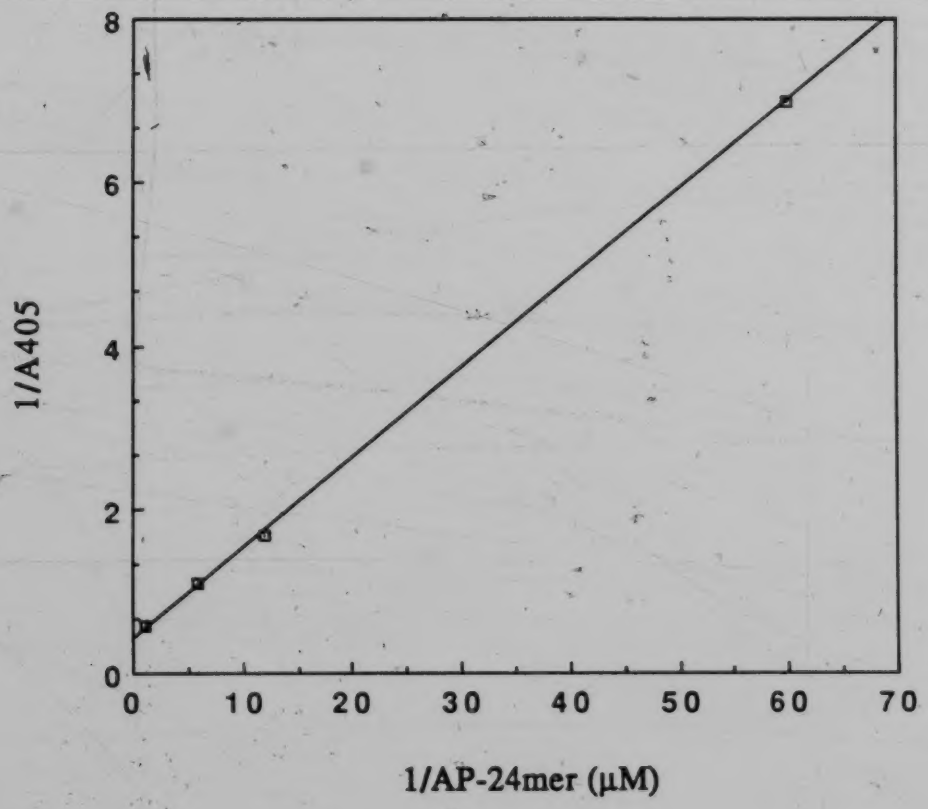


Figure VI.7. Double reciprocal plot of the binding data of Figure VI.6 The plot shows a linear line with a linear regression coefficient of 1.0.

residue synthetic peptide, corresponding to the N-terminal region of the type 1 fimbrial subunit of *E. coli*, was able to assemble into a fimbria-like structure which, when immunized in rabbits, generated antibodies which bound to type 1 fimbriae.

C. Discussion

The experiments in the present studies were designed to delineate the region of PAK pilin containing a cell-surface receptor binding domain, since no other proteins are presently known to be associated with the NMePhe pilus of *P. aeruginosa*. The results of the anti-PAK peptide antibodies revealed the C-terminal disulfide-bridged region playing a role in the binding of PAK pilus to cell-surface receptors of the buccal epithelium. Antibodies directed against both the N-terminal and the central portion of PAK pilin have no inhibitory effect on PAK pili binding to BECs. Inhibition of PAK pili binding to BECs by Fab fragments generated from antipeptide antibodies showed that only those directed against the C-terminal disulfide loop region were able to block adhesion. Of the four Fabs directed against PAK 128-144, O2 antiserum provided the best inhibition. Fabs directed against a shorter C-terminal peptide, PAK 137-144, were also able to inhibit pili binding to BECs, even though the disulfide bridge was not present. The disulfide bridge did not seem to be required for generation of inhibitory antibodies (Fab fragments) because a cysteine to alanine substitution did not affect the inhibition of pili binding to BECs. Fabs directed against other regions of the pilin molecule (outside the C-terminal disulfide loop region) did not block the adherence of pili to BECs. These results suggested that the C-terminal region of PAK pilin (residues 128-144) forms the binding domain or part of the binding domain on the PAK pili.

This conclusion was supported by studies which showed that two monoclonal antibodies, directed against the C-terminal region of PAK pilin, were also able to block adherence. The PK41C monoclonal antibody, whose epitope is in the N-terminal region, failed to block *P. aeruginosa* binding to BECs. This was in agreement with the studies

with the polyclonal antipeptide antibodies directed against the N-terminal of PAK pilin. The epitopes of the two inhibitory MAbs, PK99H and PK34C, are located at the C-terminal of the PAK pilin. The PK99H antibody was more specific than the PK34C in that PK99H only blocked PAK, and to a small degree 492c, whole cell binding to BECs. An unsual observation about PK99H was its high affinity for the reduced AcPAK 128-144 peptide even though the monoclonal antibodies were raised against native pili (with intact disulfide bridges). Two plausible explanations could account for the production of this hybridoma clone. The first explanation is that a small portion of the pilin monomers in the immunogen preparation possess cysteine residues which are in the reduced state. This small fraction of pilin monomers stimulated the production of the PK99H clone. The second explanation is that this clone was induced by pilin with oxidized cysteines since the PK99H also recognizes the oxidized AcPAK 128-144 peptides.

Earlier studies (Paranchych *et al.*, 1985) have also shown that a fragment of PAK pilin (121-144) was able to block the adherence of PAK pili to BECs. Even though the integrity of the disulfide bridge did not seem to be critical in generating anti-adhesion antibodies to PAK, it was previously shown that only the antibodies to oxidized AcPAK 128-144 were able to crossreact with PAO pili (see Chapter IV). The importance of the disulfide bridge was also illustrated by PK34C, which recognized a conformational epitope generated in part by an intact disulfide bond. The destruction of the disulfide loop also destroyed the epitope of the peptide for antibody recognition, which resulted in a loss of binding (Table VI.2). This antibody was able to block the binding of not only the PAK strain, but also the binding of all the *P. aeruginosa* strains tested. Thus, the integrity of the disulfide loop is very important in raising antibodies which are crossreactive with pili from other *P. aeruginosa* strains. The two cysteine residues on the C-terminal region are highly conserved in pilins from all *P. aeruginosa* strains. In addition, positions 131 (ser or thr), 137 (phe or tyr) and 139 (pro) are also highly conserved. The conservation of these residues within the disulfide-bridged region in the pilins of all *P. aeruginosa* strains

suggests a possible role these residues may play in maintaining a conformation conducive for receptor-binding function in the pilus. The exposure of the C-terminal disulfide-bridged region to the aqueous environment, and the importance of ser-131 in PAK pilin, were demonstrated by mutation of the PAK pilin gene (Pasloske, 1989). When ser-131 was mutated to gln-131, the binding of a PAK-specific phage, Pf (which attaches at the tip of the PAK pilus) was eliminated, suggesting the exposure of the C-terminal region at the tip of the PAK pilus.

The importance of the C-terminal region in the pilus-mediated adherence of *P. aeruginosa* was further illustrated by the binding of synthetic peptides to the cell surfaces of the buccal and tracheal epithelia. Both the reduced and oxidized AcPAK 128-144 peptides bound to the epithelial cells in a concentration-dependent manner, but only the oxidized peptides showed a saturable binding. Thus, a specific interaction was observed between the peptides and cell-surface receptors. These receptors appeared to be similar to the ones that are bound by PAK pili, because these peptides were able to compete with PAK pili for binding to BECs. This competitive inhibition demonstrated the occupation of the same sites on the cell surfaces.

It was interesting to note that in another study employing the AcPAK 116-144 peptide, an enhancement of bacterial whole cell binding to BECs was obtained. Studies by Paranchych *et al.* (1985) showed that the PAK 121-144 fragment inhibited binding of PAK to BECs. This discrepancy may be explained by the differences in the experimental conditions that were used in these studies. Previous studies by Paranchych *et al.* (1985) employed a low bacteria to BEC ratio and a high concentration of the peptide. In the present studies, a high bacteria to BEC ratio coupled with a low concentration of peptide were used and this allowed a better resolution of the pilus-receptor interaction. In order to reconcile these two differing observations, two interactions between the fragment and the receptor could be postulated since the PAK 121-144 peptide may consist of two predicted hydrophilic domains (regions 7 and 8 in Figure V.1, Chapter V; also see Sastry *et al.*,

1985): one of these interactions is a pilus-receptor interaction, and the other interaction is a pilin-pilin (subunit-subunit) interaction. A high concentration of the PAK 121-144 peptide used by Paranchych et al. (1985) may not have allowed for the resolution of the pilus-peptide (pilin-pilin) interaction thought to be observed in the present studies. The longer AcPAK 116-144 peptide completely covers the two hydrophilic domains of the C-terminal region. It can act as a bridge between the pilus and the receptor, with one domain interacting with the pilus and the other domain interacting with the receptor. The disulfide bridge is important in maintaining the function of these two domains because the destruction of the disulfide bond resulted in a loss of bacterial binding to the BECs.

Studies by Irvin and coworkers (Doig *et al.*, 1989; Irvin *et al.*, 1989) have shown that both the AcPAK 128-144 peptide and PAK pili bound to the detergent-solubilized BEC receptors which had been blotted onto nitrocellulose membrane. Polypeptide chains corresponding to molecular weights of 82, 55 to 51 and 40 kDa were observed. Furthermore, AcPAK 128-144 inhibited the binding of PAK pili to these proteins. These workers also showed that periodate oxidation destroyed the binding of both AcPAK 128-144 and PAK pili to these polypeptide chains, suggesting that the receptors are glycoproteins. Irvin *et al.* (1989) have also obtain immunofluorescence data to show that the binding of AcPAK 128-144 to TECs were localized to the cilia and luminal portions of the cell; these are the same regions to which *P. aeruginosa* whole cells were shown to bind.

The assays carried out in the above studies required the use of monoclonal antibodies and secondary antibody-enzyme conjugates to assay for binding. The times required for such assays are lengthy because of the long incubation periods. An attempt to simplify the assay has led to the use of synthetic peptides which have been conjugated directly to a reporter molecule, alkaline phosphatase. The AP-24mer conjugates (8:1 peptide to enzyme mole ratio) were able to bind to BECs with relatively high affinity (Figures VI.4 and VI.5). The relative dissociation constant of the AP-24mer was 0.94 μM compared with 6.40 μM for the oxidized AcPAK 128-144 peptide (the AP-17mer was also

assayed for binding to BECs, but the low A_{405} signal obtained with this conjugate did not allow for an accurate measurement of its affinity for cell surface receptors). These peptides have also been conjugated to horseradish peroxidase (HRP). These HRP-peptide conjugates exhibited binding properties similar to that observed with the alkaline phosphatase-peptide conjugates, in that they also bound to the PAK pili. The binding of the HRP-peptide conjugates to BECs is presently being investigated (L. Kim, K.K. Lee, R.T. Irvin, and R.S. Hodges). Preliminary studies using the AP-24mer conjugates to bind receptors looked promising (data not shown). Solubilized membrane-bound receptors from BECs immobilized on nitrocellulose membrane were lighted up using this conjugate. The bands were assayed with the normal substrates used for alkaline phosphatase in immunoblots, i.e., nitro-blue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate. Some of the bands obtained appeared to be similar to those observed by Irvin *et al.* (1989) and Doig *et al.* (1989). Inhibition studies with free peptides and PAK pili would have to be carried out to demonstrate the specificities of the conjugate-receptor interactions.

In light of the present findings, it appears that the pili-mediated adhesion of *P. aeruginosa* could be attributed to the structural pilin subunit. This mechanism is different from the pili-mediated adhesion of the *E. coli* Pap, S and type 1, where the adhesin molecule is one of the minor proteins, and not the the structural pilin subunit itself. In the Pap pilus system, papG, F and E are associated at the tip of the pilus and PapG is the adhesin molecule (Lindberg *et al.*, 1987). PapE may be an important link between PapG and the pilus filament. PapF is required for bacterial binding to receptors and may have a role in the initiation of pilus assembly. PapD acts as a chaperone (Lindberg *et al.*, 1989) and its crystal structure has been recently deduced by Holmgren and Branden (1990). The receptor for the Pap pilus in the epithelium of the urinary tract is an α-D-galactopyranosyl-(1-4)-β-D-galactopyranose digalactoside moiety in the globoseries of glycolipids (Hultgren *et al.*, 1988). The receptor for the type 1 pilus consists of a mannose moiety on protein,

while in the S fimbriae, α-sialyl-2,3-β-galactose is the receptor (Korhonen *et al.*, 1984; Parkkinen *et al.*, 1986). The type 1 pilus also has at least 3 minor tip proteins, one of which is the adhesin molecule (Hanson and Brinton, 1988; Hanson *et al.*, 1988).

In the absence of known minor proteins associated with the *P. aeruginosa* pilus, does the pilus-mediated adhesion occur at the tip or does it occur laterally with multiple receptor binding sites contributed by the many pilin subunits which make up the filamentous rod? The pilus is a contractile organelle, and bacterial adhesion occurring at the tip would not be an unusual mechanism in the adherence of bacterium to host. The contraction of the pilus would then draw the bacterium and host cell together, enabling the bacterium to adhere firmly to the surface of the host cell, leading to subsequent stages in the infection process. Such an approach by the bacterium would also bypass any repulsion of charges on the membrane surfaces of both bacterium and host cell, which would prevent a successful adhesion of the bacterium to the host cell. Immuno-gold labelling electron microscopy has been used to try to visualize protrusion of the C-terminal region at the tip of the pilus. Such an experiment has not been successful. The gold labels were neither tip located nor were they laterally distributed along the pilus length. Normally, when the pilus is coated with anti-pilus antibodies, the fuzzy appearance of the pilus would be observed. However, the use of the anti-PAK 128-144 antisera (directed against reduced or oxidized peptides) failed to give such a fuzzy appearance to the PAK pili. Preliminary data have been obtained that showed the binding of the PK99H monoclonal antibody (whose epitope is located at the region of residues 128-144 of PAK pilin) at the tip of the PAK pilus (R.T.Irvin, personal communication).

As it stands right now, both polyclonal and monoclonal antibodies directed against the C-terminal region of PAK pilin are able to block adhesion of bacteria to BECs. The peptides corresponding to the sequence of this region bind to the cell surface receptors and can competitively inhibit PAK pili from binding to them. All these data point to the structural pilin subunit containing a receptor-binding domain.

Chapter VII. Perspectives and Prospects

Despite the abundance of data presently available (Table I.1; see also Holder, 1988; Lieberman, 1985; and Pennington, 1990), the development of an anti-adhesion vaccine for *P. aeruginosa* infection has not been fully examined. Perhaps this lack of progress may have been due to an inadequate understanding of the mode and mechanism of adherence of these organisms. In order to explore and to capitalize on the potential of a peptide-based vaccine for *P. aeruginosa* infection, much research has been carried out to determine the mechanism of adherence of *P. aeruginosa* to host epithelial cells to locate the region of importance in the binding, to identify the protein involved, and to gauge the possibility of blocking this adherence process. Two adhesins are known to mediate the adherence of *P. aeruginosa* to host cells. Mucins or mucoid exopolysaccharides (MEP) mediate the adhesion of mucoid strains of *P. aeruginosa* (Ramphal and Pier, 1985; Ramphal *et al.*, 1984; Doig *et al.*, 1987). The pili-mediated attachment of nonmucoid strains to mammalian epithelial cells (Doig *et al.*, 1988; Ramphal *et al.*, 1984; Sato and Okinaga, 1987; Woods *et al.*, 1980). Purified pili from a particular strain were able to block adherence of homologous strains. Similarly, antibodies against pili were only able to block the binding of homologous strains (Ramphal *et al.*, 1987; Woods *et al.*, 1980). Ramphal *et al.* (1987) reported that an antiserum against MEP from a single mucoid strain inhibited the adherence of all six mucoid isolates tested (from CF patients), suggesting antigenic similarities of the MEP of mucoid strains. However, using more refined binding studies, Doig *et al.* (1987) found that alginates from various strains of *P. aeruginosa* possessed structural diversities. Alginate-based vaccines have had only limited success (Woods and Bryan, 1985). Immunization with purified alginate resulted in bacterial clearance which was associated with an increase in anti-alginate antibody titer. This protection was strain-specific in the chronic lung infection with *P. aeruginosa* in rats. However, it was also found that the anti-alginate antibodies formed immune complexes and resulted in tissue damage. This

undesirable damaging effect makes the alginate an unsuitable vaccine candidate. However, ongoing research is being carried out to improve the alginate vaccine (Garner *et al.*, 1990; Pier *et al.*, 1987; Pier *et al.*, 1990). It is believed that the appearance of the alginate producing mucoid strains emerge after initial colonization by nonmucoid *P. aeruginosa* strains (Dogget, 1969; Hoiby, 1974). Clearly, the prevention of colonization by pilus-mediated nonmucoid strains would be a better target for developing an anti-adhesion vaccine. The documented evidence that antibodies against pili could block adherence, albeit of homologous strains (Ramphal *et al.*, 1987; Woods *et al.*, 1980), offered hope for a pilus-based vaccine which would interrupt the initial colonization process.

To delineate the region of pilus involved in the pilus-mediated adherence, the project was designed to map the surface regions of pilin and from there determine which surface region/s contributed to the binding properties of pilus. This rationale was based on the premise that no other minor proteins are associated with the filamentous rod which could contribute to the adhesive property of pilus. Pili from strains PAK and PAO were selected for the studies because they have been characterized and purified (Paranchych *et al.*, 1980; Pasloske *et al.*, 1985; Sastry *et al.*, 1983; Sastry *et al.*, 1985).

The predicted surfaces of PAK and PAO pilins based on the Surfaceplot program (Parker *et al.*, 1986) yielded similar surface profiles and a possibility of eight surface regions on both PAK and PAO pilins (Figure V.1, Chapter V). In fact, when the prediction algorithms were applied to three other pilins, P1, CD4 and K122-4, similar surface profiles were observed (Pasloske *et al.*, 1988; unpublished data). In comparative studies carried out by Dalrymple and Mattick (1987), the conservation of certain regions of the NMePhe pilin is maintained in several genera, including *P. aeruginosa*, *N. gonorrhoeae*, *B. nodosus* and *M. bovis*. Since these organisms share the same class of NMePhe pili, they have very similar morphology, i.e., 5 μm in length and 0.05 μm in diameter. Within the same *Pseudomonas* species, pilin molecules from different strains would be expected to have similar structural features. The highly conserved N-terminal

region (residues 1-30) believed to be involved in pilin-pilin interaction is supported by the physicochemical studies of Watts *et al.* (1983). Mutation in the first few residues at the N-terminus and also the disruption of the methylation process of the N-terminal phenylalanine resulted in unassembled pilin subunits (Pasloske *et al.*, 1988). Other conserved residues in the pilin molecule may also participate in pilin-pilin interactions.

In the mapping studies, most of the predicted surface regions were located by antipeptide antisera raised against synthetic peptides corresponding to those regions predicted by the Surfaceplot program (Chapter V), thus showing its effectiveness. In one or two cases, the antipeptide antiserum failed to bind to pili. However, antiserum raised against whole protein was able to bind to these synthetic peptides, attesting to their surface location and to their immunogenicities and antigenicities (e.g. PAK 74-83). Regions on the surface need not be immunogenic, e.g., PAK 88-97 and PAO 88-97, when immunized as part of the native protein. However, immunization with the peptide conjugated to a protein carrier, resulted in good humoral response to this region of the protein. This is part of the elegance of using a peptide immunogen. Regions on a protein which are ordinarily immunorecessive can be made to induce antibody production with a specificity just to the peptide or region of interest. The limitation is that the ability of the antipeptide antibodies to bind to native protein may not be of a high affinity because of conformational constraints of that region of the protein. Thus, there are researchers who would make synthetic peptides based on secondary structure predictions because it is thought that antibodies bind to β-turns. β-turns structures tend to be found on the surface of proteins. The antigenic sites often have convex surface shapes, while the complementary binding sites on the antibodies have concave shapes (Geysen *et al.*, 1987). However, our understanding as to why peptides can generate antibodies which bind to proteins is still limited. Generally, peptides do not have well defined structures in solution and yet are able to generate antibodies that bind the native proteins. The perplexity of this 'ordered-disordered paradox' was again highlighted by recent crystallographic data on the binding of an antipeptide monoclonal

antibody to the peptide and its corresponding native protein (Stanfield *et al.*, 1990). A synthetic 19-residue peptide homolog of the C-helix of myohemerythrin (Mhr) was cocrystallized with a monoclonal antibody specific for the first five residues of this peptide. This region of the peptide was shown to be absent of any regular structure in solution by NMR and CD measurements. It was found that the peptide adopted a type II β-turn conformation when bound to the Fab fragment, showing that the antibody bound to the peptide in a well-defined secondary structure common in proteins. The binding of this monoclonal antibody to the native Mhr would be interesting because the same sequence on the protein has been shown to adopt an α-helical structure in the native protein (Sheriff *et al.*, 1987), quite different from the solution structure or the Fab-bound structure. Furthermore, one of the essential residues (His-73) which contributed to the epitope was intimately involved in the coordination of an iron atom internally and would not be available for binding to the antibody. Some denaturation of the native protein would be anticipated for binding to occur, and in fact the apo-form of the protein did bind much better to the Fab than its native form. Further light on the mechanism of antigen-antibody binding in this example will have to wait until these researchers have successfully crystallized the Fab-apo Mhr complex, as well as the complexes of two other monoclonal antibodies to the 19-residue peptide.

Although it was possible to obtain crossreactive and strain-specific antibodies from the predicted surface regions, the ability to predict crossreactivity and strain-specificity in related proteins appears to be more than just a simple comparison of the primary amino acid sequences of the proteins. Structural features inherent in the primary sequence may have a role in determining whether the region of the molecule would be crossreactive or strain-specific in related proteins.

The conservation of residues in the semiconserved C-terminal region of *Pseudomonas* pilin is important in the functionality of this molecule. The region around residues 121-144 in PAK pilin is immunogenic but it is not the immunodominant region,

which is thought to be a conformational epitope located between residues 82-110 (Sastry *et al.*, 1985; Watts *et al.*, 1983). Anti-pilus antisera against PAK and PAO pili can recognize synthetic peptides corresponding to residues 128-144 of the protein. Immunization of mice with PAK pili gave rise to monoclonal antibodies which could bind the disulfide-bridged region of PAK pilin. This disulfide-bridged region contains a receptor-binding domain, as evident by the abilities of both polyclonal and monoclonal antibodies to bind this region, and subsequently block both pili and bacteria from binding BECs. The immunorecessiveness of this region would play an important role in that it would be less likely to be bound by host antibodies which would block its adherence function. It is a common ploy for microorganisms to divert the host defence system away from the important functional regions. The immunodominant region of PAK pilin is located away from the C-terminal region. Thus, polyclonal antipeptide antibodies directed against regions away from the C-terminal region, such as the region of residues 88-97, 105-114 and 117-125, did not block PAK pili binding to BECs. The antibodies, anti-PAK 128-144, PK99H and PK34C, bound and blocked adherence of both pili and bacteria to BECs. The two cysteine residues are conserved in all the known *P. aeruginosa* pilin sequences. In addition, there are other conserved residues in this region such as Ser-130, Phe-137 and Pro-139 (Figure I.1, Chapter 1) which may contribute to receptor-binding function.

The conservation of the two cysteines at the C-terminal region of the NMePhe pilin and their forming a putative disulfide bond has led to speculations of a possible role for such a disulfide bond in bacterial pilus function. Within this loop region lies a strain-specific immunodominant epitope of gonococcal pilin (Rothbard *et al.*, 1984). Although controversial, the hypervariable disulfide loop region may also contain part of the receptor-binding domain of gonococcal pilus (Nicholson *et al.*, 1987). As stated by Schoolnik *et al.* (1983), if each pilus subunit possesses a receptor-binding domain, one would expect a polyvalent ligand with a linear array of binding sites along its longitudinal axis. This region of the molecule could be involved in pilin-pilin interactions and, thus, would not be

exposed along its longitudinal axis. If so, only the distal pilin subunit would be exposed at the tip of the pilus, leading to a single receptor-binding domain for each assembled pilus. Based on prediction algorithms used by Schoolnik *et al.* (1984), the C-terminal disulfide bridge region is fairly hydrophilic and could be exposed to the aqueous environment. This exposure would then enable this region to act as a receptor-binding domain. As mentioned earlier, no minor proteins associated with the gonococcal pilus or with other NMePhe pili have been discovered. The pilin molecules do not undergo post-translational addition of carbohydrate, sulfate or phosphate groups which may contribute to binding activities. However, recent reports by Muir *et al.* (1988) and Parge *et al.* (1990) suggested that other proteins may be associated with the gonococcal pilus. These putative pilus-associated proteins have not been characterized nor are their functions on the pilus known. The studies carried out with the PAK pilin demonstrated that the C-terminal disulfide-looped terminal region (PAK 128-144) contains a receptor-binding domain for the PAK pilus. Polyclonal antibodies to this peptide blocked the adherence of pili to BECs. Monoclonal antibodies directed against this region of residues 121-144 block both homologous (PAK) and heterologous strains from binding to BECs. Moreover, the direct binding of the AcPAK 128-144 peptide to cell-surface receptors demonstrated unequivocally that this region of pilin is a receptor-binding domain on the PAK pilin. It will also be important to verify these results with the binding of synthetic peptides, corresponding to the C-terminal region of pilins from the other *P. aeruginosa* strains, to cell-surface receptors. Antibodies raised against these peptides can also be used to block *Pseudomonas* adherence to host cells.

The prospect of a synthetic peptide vaccine for *P. aeruginosa* infection from an anti-adhesive approach looks very promising. We have been able to locate a region of the *Pseudomonas* pilin which contains a receptor-binding domain and antibodies directed against this region can block bacterial adherence. Rowlands (1989) stated two basic requirements for a peptide vaccine: firstly, a sequence of amino acids capable of eliciting

antibodies which react with the native protein from which the sequence was derived (B-cell site); and secondly, a sequence of amino acids which could induce T-cell help for the B-cell. The first requirement has been met in the present research for a synthetic peptide vaccine for *P. aeruginosa* infection. However, the second issue has not been dealt with in this thesis. The humoral response requires cooperation between B and T lymphocytes. The B and T cells are specialized cells in the immune system. The proliferation and maturation of B-cells require not only the stimulatory signal from the antigen, but also from a T-helper cell. The T-cells are primed to proliferate and mature into helper cells after exposure to an antigen, in combination with major histocompatibility complex molecules on antigen-presenting cells. The T-cell epitopes are usually, but not always, different from those of B-cell epitopes. The reason for the use of large protein carriers such as KLH is that it has a number of T-cell epitopes which help stimulate a helper T-cell response. Enhancement of the immunogenicity of the vaccine could be achieved by the incorporation of B- and T- cell epitopes onto the same carrier molecule. Although the presence of the carrier may have undesirable side-effects and may abrogate some of the potential advantages of a totally synthetic vaccine (Rowlands, 1989), suitable human carrier proteins, such as tetanus and diphtheria toxoids, are applicable. The use of these toxoids in humans has been approved by the Food and Drug Administration. Alternatively, instead of using a monomeric presentation, polymers of the B-cell epitope could be employed to enhance the humoral response if a protein carrier is not desirable. In addition to the above approaches, a totally synthetic method of presenting peptides as regular polymeric arrays involving the use of a synthetic core consisting of polymers lysine residues, has been developed by Tam (1988). In this multiple antigen peptide (MAP) approach, peptide antigens are incorporated via the α- and ξ- amino groups of the lysine residues in the core structure, to give rise to a macromolecular structure without the presence of a large protein carrier. Such an approach has been extended to include both B- and T- cell epitopes (Tam and Lu, 1989). Another issue with the efficacy of synthetic peptide vaccines is that short

linear peptides are often insufficient for generating good immunological responses in a divergent genotypic population. To ensure an immune response in an outbred population, the vaccine may have to be incorporated with a cocktail of helper T-cell epitopes. The issue of cell-mediated immunity has not been dealt with in this thesis. From previous studies (Smart *et al.*, 1988), the major T-cell epitope of the *P. aeruginosa* PAK pilin has been located in the region of residues 82-104. Further studies which have been carried out to pinpoint this sequence to a linear 15-residue sequence have not been successful (B. Singh, K.K. Lee, and R.S. Hodges, unpublished data), although studies are still being carried out to map the T-cell epitopes of PAK pilin (B. Singh, K.K. Lee, R.S. Hodges, and A. Holm). Delineation of the major T-cell epitope may enable us to enhance the immunogenicity of a pilus-based synthetic peptide vaccine.

The important contribution of each amino acid residue in the receptor-binding region of *P. aeruginosa* pilin should be examined. In this regard, studies are underway in this laboratory to synthesize single amino acid (alanine) substitutions in the region of residues 121-144 of PAK pilin, with the exception of the two cysteines to conserve the disulfide bridge in all the analogs. These analogs will be used in two ways. Firstly, they will be used to map the epitopes of the strain-specific PK99H MAb and the crossreactive PK34C MAb. In addition, the mapping of the epitopes of the crossreacting (17-O1) and the strain-specific (17-O2) polyclonal antibodies is presently being pursued in this laboratory (W.Y. Wong and R.S. Hodges). The data from these studies should provide insight into the residues that contribute to strain-specific and crossreacting antibodies. Secondly, the analogs will be used for binding studies to determine which residues, when substituted, would contribute to a significant loss of binding. This would enable us to know which of the residues in the C-terminal region contribute to receptor-binding function and how widely conserved these residues are in the pilins from different *P. aeruginosa* strains. By substituting all the residues not involved in adherence with alanine or glycine residues, perhaps a better crossreacting antibody could be obtained. Alternatively, a

cocktail vaccine using the C-terminal regions of all pilin types and conjugated to a carrier molecule may also be tested. In collaboration with Drs. Randy Read and Randy Irvin (Medical Microbiology and Infectious Disease, University of Alberta), crystallization of the AcPAK 128-144 and AcPAK 121-144 peptides and the co-crystallization of the monoclonal antibodies with these peptides are being undertaken to rationalize the structure of the peptides and its binding to the antigen-binding site of the antibodies. Such an approach should provide a more rational way to designing a *Pseudomonas* vaccine.

Another area of study which should be pursued is whether there are other minor protein components (besides the pilin subunits) associated with the PAK pilus. In the Pap pilus of *E. coli*, the adhesin molecule along with other pilus-associated proteins are located at the tip (Lindberg *et al.*, 1984; Lindberg *et al.*, 1987; Lund *et al.*, 1987), while in the type 1 pilus, the adhesin and other pilus-associated proteins are distributed along the shaft (Hanson *et al.*, 1988; Krogfelt *et al.*, 1990; Maurer and Orndoff, 1987). No other minor proteins associated with the *Pseudomonas* pilus have been found. The absence of available evidence does not necessarily negate their existence in the NMePhe pilus system. The approach to this problem of whether there are other pilus -associated proteins may lie in genetic studies to localize the pilin operon of *P. aeruginosa*. Three other genes involved in *P. aeruginosa* pilus expression have been reported (Nunn *et al.*, 1990). Further studies to delineate the pilus operon are underway. In this way, the genetic approach coupled with DNA sequencing studies and biochemical characterization of the gene products may shed some light on the NMePhe pilus system of *P. aeruginosa*.

Two interesting developments were initiated in the course of the present research project. The first involved a new monitoring procedure for reduced (free sulfhydryl groups) and oxidized (intrachain disulfide bonded) peptides using RPC. Peptides to be oxidized to form intrachain disulfide bond could be modified with N-ethylmaleimide (NEM) and separated by RPC. This development came about as a result of the inability to monitor clearly the reduced and oxidized AcPAK 128-144 peptide by RPC in the early

phase of the research. This new method provided good baseline resolution between reduced and oxidized conformers. This is particularly useful for peptides that are not resolved by RPC. This method is not as fast as the colorimetric Ellman assay (Ellman, 1959). Unlike the Ellman method which monitors the free sulfhydryls alone, this RPC method monitors the UV absorbance of both reduced and oxidized peptides. It was found to be highly sensitive and only small quantities of peptides (nanomolar range) were needed. It is possible to monitor both reduced and oxidized peptides using electrochemical detectors which are very sensitive detector systems (Allison and Shoup, 1983). The application of electrochemical detectors system for thiol-containing peptides has been limited. This sensitive detector system has been recently reviewed by Dou *et al.* (1990). The simplicity of the RPC method negates the unnecessary purchase of such detectors in routine monitoring and quantitation of reduced and oxidized peptides. This method is now routinely used in this laboratory to monitor the oxidation of peptides.

The second development was the use of ligand-reporter conjugates to assay protein-receptor or protein-protein interactions. Although this approach is still in its infancy, the use of the alkaline phosphatase-PAK 121-144 conjugate (AP-24mer) to bind to pili and cell-surface receptors has shown great promise. This is not a new idea as such because many ligands have been conjugated to reporter molecules and used in other biological assays. The side-chains of the amino acids, such as the ξ-amino, α- and β- carboxyls and the thiol groups, are useful for chemical modifications because the reactivities can be pH-controlled and many heterobifunctional reagents have been designed to react with these side-chains to generate hetero-oligomers. The chemical aspects and application of protein modification have been recently reviewed by Means and Feeney (1990). An excellent source of these bifunctional reagents is the Pierce Chemical Company catalogue. Some of the practical aspects of preparation of protein-conjugates have been reviewed by Thorpe and coworkers (Cumber *et al.*, 1985) and Koppel (1990). The conjugation method developed in the present study uses a simple photoreactive molecule attached at a defined

site on the peptide during synthesis of the peptide (Parker and Hodges, 1985). Because the photolytic crosslinking of the benzoylbenzoyl moiety is non-specific in nature in that it does not rely on functional side chains of amino acids, the peptide could easily be covalently crosslinked to the surface of the reporter molecule upon irradiation with UV light. The activity of the reporter molecule may be reduced because of steric hindrance due to a covalently-attached peptide close to the cleft of the active site. The attachment of peptide to reporter molecule was non-specific and as such a loss of activity could not be tightly controlled but the probability of this occurrence in a given population should be statistically low. Further reduction of the loss of enzymatic activity could be achieved by controlling the number of peptides to be conjugated to each reporter molecule. The application of this methodology was demonstrated in the binding of the AP-24mer to immobilized PAK pili on the wells of the ELISA plates and to cell-surface receptors on BECs. Thus, the study of protein-protein and ligand-receptor interactions of many biological systems could be carried out in this way if the region of interaction is contained within a linear sequence of the protein. One half of the bimolecular process could be immobilized to a solid support and the second half (ligand) conjugated to an enzyme or other reporter molecules. The binding process could be visualized and quantitated spectrophotometrically. Characterization of the binding could be determined with peptide analog inhibition assays and many such assays could be carried out in multi-well polystyrene plates. Another application could be the isolation of receptors. Receptors isolated by gel electrophoresis could be visualized by the AP-24mer conjugates. This assay could facilitate isolation of receptors which are membrane-bound or cytosol-localized, e.g., peptide hormone receptors.

Peptide inhibition studies using the AP-24mer binding to BECs and to PAK pili will have to be carried out to show the specificities of this assay. Quantitation of the molar ratios of peptide to alkaline phosphatase needs to be carried out to determine the optimum ratio which would give high affinity and high sensitivity of the conjugate with minimal loss of enzymatic activity or loss of binding. The question of the length of the spacer arm

should be considered. In the conjugates that were made, the length of the spacer arm between the peptide and the alkaline phosphatase was made up of the photoreactive benzoylbenzoyl moiety and the norleucine residue. The fact that the AP-17mer bound poorly to PAK pili and to BECs could be due to the close proximity between the binding domain on the peptide and the enzyme surface which resulted in steric hindrances between the peptide and the receptor. Thus, the appropriate length of the spacer arm should be established. The viability of this method should be tested in other biological systems. One such assay might be the immobilization of the anti-PAK 128-144 immunoglobulin or Fab to the wells of the ELISA plate and assaying the binding of the AP-24mer conjugate to these protein receptors. The further development and establishment of this methodology could provide other scientists involved in many areas of ligand-receptor and protein-protein interaction research with a new powerful tool to unravel some of the mechanisms of the biological world.

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